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THE EFFECT OF ORAL HYPOGLYCEMIC AGENTS ON THYROID FUNCTION IN THE RAT[1]

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ABSTRACT

In the rat carbutamide and, to a much lesser extent, metahexamide result in reduction in thyroidal I^{131} uptake and I^{127} concentration. Only carbutamide is actually goitrogenic. In comparably hypoglycemic doses tolbutamide, chlorpropamide and phenethylbiguanide do not show antithyroid activity.

THE administration of carbutamide has been shown to result in an increase in thyroid weight, a decrease in radioiodide uptake and depletion of glandular iodine stores in the rat thyroid gland (1, 2). Its mechanism of action appears to be similar to that of propylthiouracil, although its potency as an antithyroid agent in the rat is only 1/200th as great (1). In the human, carbutamide also produces mild but definite antithyroid effects (3, 4). Tolbutamide and chlorpropamide, which are closely related chemically to carbutamide, induce slight and transient antithyroid effects in the human (4, 5), but during long-term administration to diabetic patients, no instances of the production of hypothyroidism or goiter have as yet been reported. Metahexamide administration has resulted in no change in measures of thyroid function in the human (6).

In view of the definite antithyroid activity of carbutamide in the rat, it appeared of interest to determine if other hypoglycemic agents shared this property. In the present study, tolbutamide, chlorpropamide, metahexamide, and phenethylbiguanide were examined for their effects on the thyroid gland of the rat.

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MATERIALS AND METHODS

In the first experiment immature Sprague-Dawley rats weighing 100–120 gm. were changed from Purina chow to the synthetic low iodine diet of VanderLaan and Caplan (7). Chemical agents were ground in a mortar with a small quantity of the diet and mechanically mixed into the diet. Rats were divided into groups of six or seven and fed a designated diet *ad libitum* for ten days as listed in Table 1. The quantity of diet consumed was not measured. The estimated food intake is in the order of 10 grams per rat per day, or less. Using this figure as a basis for calculation, the dosage of drugs used, in terms of mg./kg./day was equal to or less than the following: .05% = 50 mg./kg./day; .1% = 100 mg./kg./day; .125% = 125 mg./kg./day; .25% = 250 mg./kg./day; .5% = 500 mg./kg./day; 1.5% = 1,500 mg./kg./day; 2.5% = 2,500 mg./kg./day.

For estimation of thyroid uptake of I^{131}, approximately 4 microcuries of I^{131} were injected intraperitoneally on the ninth day of the diet, twenty hours prior to death. Rats

TABLE 1. EFFECTS ON THYROID FUNCTION OF HYPOGLYCEMIC AGENTS FED TO RATS IN AN *ad lib.* DIET

Diet	No.	Sex	Body weight, gm.	P	Thyroid weight, mg.	P	Thyroid I^{127}, mg./100 gm. thyroid weight	I^{131} uptake % of administered dose	P
Control	7	M	124.6±4.2*	—	6.99±.30*	±	46.87	16.8±1.5*	—
Chlorpropamide									
0.25%	7	M	113.9±2.3	<.05	7.83±.44	NS	23.35	22.4±1.9	<.05
0.125%	7	M	111.7±3.0	<.05	8.69±.56	<.02	22.53	25.6±1.8	<.01
0.05%	7	M	126.6±4.1	NS**	9.50±.48	<.001	17.42	23.5±3.3	NS
Phenethylbiguanide									
0.5%	6	M	80.8±3.8	<.01	4.71±.26	<.001	49.56	24.2±1.9	<.01
0.25%	6	M	95.7±3.7	<.01	5.73±.50	NS	50.97	28.6±2.6	<.01
0.1%	6	M	93.2±6.1	<.01	6.00±.12	<.02	35.71	26.7±2.6	<.01
Metahexamide									
0.25%	6	M	95.7±5.3	<.01	6.52±.39	NS	18.08	19.4±2.1	NS
0.125%	6	M	104.8±3.4	<.01	6.10±.45	NS	23.49	22.7±2.7	NS
0.05%	6	M	114.6±1.8	NS	6.45±.51	NS	24.20	29.3±3.2	<.01
Control	8	F	98 ±3.5	—	6.17±.24	—	33.16	15.8±1.1	—
Carbutamide 0.5%	7	F	92.9±4.6	NS	14.75±1.32	<.001	3.05	5.12±2.1	<.01
Tolbutamide									
2.5%	7	F	86.9±3.1	<.05	5.96±.14	NS	36.17	21.9±1.4	<.01
1.25%	7	F	90.4±3.2	NS	5.67±.25	NS	40.40	24.8±4.5	NS
0.5%	7	F	101.7±3.1	NS	6.53±.37	NS	32.80	20.4±2.0	NS

* Mean ± Standard Error of the Mean.
** NS = P > .05.

were killed with chloroform and the thyroid glands were removed, weighed, and counted. The thyroid I^{127} content was determined on the pooled thyroid glands of each group by the alkaline ash method of Barker *et al.* (8).

The rats used in the second experiment were tube fed with calorically equal feedings of a synthetic diet modified from that of Whitney and Roberts (9), containing approximately 20% protein, 20% fat, and 60% carbohydrate by weight. All animals were fed 4 cc. twice daily for three days and then 5 cc. twice daily for the remainder of the experiment. Two and one-half per cent solutions of metahexamide and chlorpropamide were made up in 0.05N NaOH. An 0.75% aqueous solution of phenethylbiguanide and a 1.5% aqueous solution of the sodium salt of tolbutamide were prepared, and propylthiouracil was made up in 0.05% aqueous solution with ½ drop of Tween-80 per 20 ml. One half ml. of each of the above solutions was injected subcutaneously twice daily at 8:00 A.M. and 5:00 P.M. providing a total daily dose averaging 250 mg. per kg. of chlorpropamide and metahexamide, 75 mg. per kg. of phenethylbiguanide, 1,500 mg. per kg. of tolbutamide,

and 5 mg. per kg. of propylthiouracil. These dosages were determined as productive of maximal hypoglycemic action in the range believed to be sublethal as suggested by the work of Ungar *et al.* (10) and Root *et al.* (11). I^{131} was administered as in the first experiment on the tenth day of the diet and I^{131} and I^{127} determinations were performed in the same manner except that individual assays for I^{127} content were performed on the total thyroid gland of each animal. Six rats were included in each group initially, but a few deaths occurred early in the tube-feeding regimen, all due directly to feeding accidents.

RESULTS

The results of Experiment 1, in which the drugs were administered by *ad lib.* feeding, are given in Table 1. Carbutamide at a dietary concentration of 0.5% led to the expected decreases in thyroid I^{131} uptake and I^{127}

TABLE 2. EFFECTS ON THYROID FUNCTION OF HYPOGLYCEMIC AGENTS ADMINISTERED SUBCUTANEOUSLY TO TUBE-FED RATS

Drug	No.	Weight gain, gm.	P	Thyroid weight, mg.	P	Thyroid I^{127} mg./100 gm. thyroid weight	P	I^{131} uptake % of administered dose	P
Control	6	27.5±2.2*	—	6.68± .65*	—	56.6 ±2.28*	—	5.45± .55*	—
Chlorpropamide 250 mg./kg./d.	3	22.7±3.5	NS**	6.14± .42	NS	44.7 ±3.13	<.01	7.03± .70	NS
Phenethylbiguanide 5 mg./kg./d.	5	28.0±1.7	NS	6.94± .26	NS	53.9 ±6.61	NS	6.11± .49	NS
Metahexamide 250 mg./kg./d.	6	25.7± .7	NS	6.82± .52	NS	28.3 ±1.94	<.001	3.88± .38	<.01
Tolbutamide 1500 mg./kg./d.	6	26.5±2.6	NS	6.55± .33	NS	34.6 ±2.60	<.001	5.05± .64	NS
Propylthiouracil 5 mg./kg./d.	5	30.8±1.5	NS	26.04±1.07	<.001	0.37± .04	<.001	0.89± .04	<.01

* Mean ± Standard Error of the Mean.
** Ns = P >.05.

concentration and the expected increase in thyroid weight. However, significant increases over that of the controls were observed in thyroid I^{131} uptake of rats ingesting various dosage levels of chlorpropamide, phenethylbiguanide, metahexamide and tolbutamide. The stimulation of I^{131} uptake was generally correlated with failure to gain weight and an unhealthy appearance of the rats. Thyroid I^{127} concentration was significantly reduced by all three dosage levels of chlorpropamide and metahexamide. Alterations in thyroid weight were minimal in all groups.

The results of Experiment 1 suggested that food intake and therefore the dose of drug may have varied among the different experimental groups. Accordingly, in Experiment 2, food intake was held constant by equal tube feedings and drugs were administered subcutaneously in large doses. Groups of rats were matched for average body weight prior to the start of the experiment. Under these conditions, body weight gain was equal in all groups and no significant *increases* in thyroidal I^{131} uptake were noted (Table 2). On the other hand, propylthiouracil in a daily dose of 0.5 mg. per rat produced a marked fall in I^{131} uptake; metahexamide caused a lesser but significant fall. Only propylthiouracil led to significant

increase in thyroid weight. However, metahexamide and tolbutamide caused significant depletion of thyroidal iodine concentration, although not to as great an extent as propylthiouracil.

DISCUSSION

The predictable effects of a goitrogen on the parameters of thyroid function which we examined are a decrease in the 20-hour I^{131} uptake, an increase in thyroid weight and a decrease in the thyroid I^{127} concentration. Such a pattern was clearly seen when propylthiouracil or carbutamide were administered. In Experiment 1, other oral hypoglycemic agents appeared to be associated with decreases in thyroid I^{127} concentration. However, I^{131} uptake was increased, body weights were subnormal and the animals appeared ill. It is suggested that this response may have been due largely to the toxicity of the drugs with resultant decrease in dietary

TABLE 3. A COMPARISON OF THE SIGNIFICANT EFFECTS (AT THE 5% LEVEL) ON THYROID FUNCTION OF SIMILAR DOSES OF THE HYPOGLYCEMIC AGENTS ADMINISTERED ORALLY *ad lib.* WITH A LOW IODINE INTAKE AND GIVEN SUBCUTANEOUSLY WITH A NORMAL IODINE INTAKE AND FORCED FEEDING

Drug	Route	Dose (mg./kg./d.)	Dietary iodine	Body weight	Thyroid weight	Thyroid I^{127} content	Thyroid I^{131} uptake
Chlorpropamide	Oral	250	Decreased	Decreased	No Change	Decreased	Increased
	Subcutaneous	250	No Change	No Change	No Change	Decreased	No Change
Phenethylbiguanide	Oral	100	Decreased	Decreased	Decreased	Decreased	Increased
	Subcutaneous	75	No Change	No Change	No Change	No Change	No Change
Metahexamide	Oral	250	Decreased	Decreased	No Change	Decreased	No Change
	Subcutaneous	250	No Change	No Change	No Change	Decreased	Decreased
Tolbutamide	Oral	1,250	Decreased	No Change	No Change	No Change	No Change
	Subcutaneous	1,500	No Change	No Change	No Change	Decreased	No Change

I^{127} intake, which would be critical because of the low concentration of iodine in the diet. The lack of significantly increased thyroidal I^{131} uptake in the .25% and .125% metahexamide groups may be explained by the mild anti-thyroid effect demonstrated for this drug in the second experiment. That the majority of changes noted in Experiment 1 were effected chiefly by the decreased iodine intake is more apparent if one compares the effects of similar doses of these drugs in the two experiments as in Table 3.

In Experiment 2, on the other hand, intragastric administration of calorically equal and nutritionally adequate feedings resulted in healthy animals of normal weight despite very large doses of the various agents. In this experiment, metahexamide produced both depression of I^{131} uptake and depletion of I^{127} content and thus appeared to be a mild antithyroid agent, although not goitrogenic under the conditions of this experiment. The absence of an increase in thyroid weight may be explained by the fact that this measurement is a less sensitive index of thyrotropin action than are the I^{131} uptake and I^{127} concentration (12). Another possible factor is

The antigoitrogenic effect of increased dietary iodide in the presence of antithyroid drugs, demonstrated for the thiourea group by McGinty (13) and extended to carbutamide in previous work from this laboratory (1). The tube feeding administered in Experiment 2 contained sufficient iodine to result in a thyroidal I^{131} uptake in control animals of 5.4%, as compared to 16.8% in the control animals ingesting a low iodine diet in Experiment 1. The greater I^{127} concentration in the thyroid glands of control rats in Experiment 2 confirms this assumption. On the other hand, the increase of dietary iodine was not sufficient to obscure the highly potent goitrogenic action of propylthiouracil under the same experimental conditions.

Whereas metahexamide thus appears to possess mild antithyroid activity, tolbutamide has only equivocal effects, phenethylbiguanide has none and chlorpropamide probably has none, although conclusions regarding the latter drug are tentative because of the small number of animals surviving. The anti-thyroid activity of carbutamide is confirmed in the present experiments; in comparably hypoglycemic doses it appears to be a far more potent goitrogen than metahexamide or tolbutamide. This indicates that antithyroid activity of members of this group of agents is not correlated with hypoglycemic potency, as previously suggested by Wrenshall (2).

REFERENCES

1. Brown, J. and D. H. Solomon: *Endocrinology* **63**: 473. 1958.
2. Wrenshall, G. A.: *Ann. N. Y. Acad. Sci.* **71**: 164. 1957.
3. Brown, J. and D. H. Solomon: *Metabolism* **5**: 813. 1956.
4. McGavack, T. H., W. Seegers, H. O. Haar, J. Enzinger and V. O. Erk: *Ann. N. Y. Acad. Sci.* **71**: 268. 1957.
5. Skinner, N. S., Jr., R. L. Hayes and S. R. Hill, Jr.: *Ann. N. Y. Acad. Sci.* **74**: 830. 1959.
6. Hamwi, G. J., T. G. Skillman, L. R. Freedy and L. A. Alexander: *Metabolism* **8**: 631. 1959.
7. VanderLaan, W. P. and R. Caplan: *Endocrinology* **54**: 437. 1954.
8. Barker, S. B., M. J. Humphrey and M. H. Soley: *J. Clin. Investigation* **30**: 55. 1951.
9. Whitney, J. E. and S. Roberts: *Am. J. Physiol.* **181**: 446. 1955.
10. Ungar, G., L. Freedman and S. L. Shapiro: *Proc. Soc. Exper. Biol. and Med.* **95**: 190. 1957.
11. Root, M. A., R. C. Anderson and J. S. Welles: *Metabolism* **8**: 565. 1959.
12. Ghosh, B. N., D. M. Woodbury and G. Sayers: *Endocrinology* **48**: 631. 1951.
13. McGinty, D. A.: *Ann. N. Y. Acad. Sci.* **50**: 403. 1949.

VASOPRESSIN STIMULATION OF THE ISOLATED ADRENAL GLANDS: NATURE AND MECHANISM OF HYDROCORTISONE SECRETION[1,2]

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ABSTRACT

By means of direct arterial perfusion of the adrenal glands of the hypophysectomized dog, it has been shown that synthetic lysine, arginine and acetyl arginine vasopressins stimulate the adrenal cortex directly to secrete hydrocortisone. Maximal cortisol secretion occurred at dose ranges of 100 to 400 milliunits per minute, with minimal activity being detected with values as low as 1 milliunit per minute of arginine vasopressin. At maximal dosages the response was comparable to that seen following 1 unit per minute of ACTH. Pressor activity and cortisol stimulating activity were demonstratrated to be independent to each other. In addition similar polypeptides such as oxytocin, insulin and glucagon and pressor amines such as epinephrine and norepinephrine did not show any cortisol stimulating activity.

ACTH was consistently found to increase aldosterone secretion when perfused through the glands of hypophysectomized animals.

The probable role of arginine vasopressin as a factor in the stress reaction is discussed as well as its posulated ability to activate adrenal phosphorylase.

RECENTLY it has been proposed that vasopressin, formed in the median eminence of the ventral hypothalamus, may be responsible for the activation of the adenohypophysis and the release of adrenocorticotropic hormone (ACTH) into the circulation (1, 2, 3). In support of this hypothesis is the finding that pitressin increases the level of ACTH in the blood (4), the fact that antidiuretic hormone (ADH) is released into the circulation of animals exposed to noxious stimuli (1), the observed defect in ACTH release in rats with diabetes insipidus due to hypothalamic lesions (5) and the rise in blood 17-hydroxycorticoids seen following the administration of pitressin or purified arginine vasopressin to humans (6, 7).

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However, it has been shown that in hypophysectomized dogs (8), as well as in hypophysectomized rats (9), the intravenous injection of vasopressin causes a rise in 17-hydroxycorticoids in adrenal venous blood and a depletion of adrenal ascorbic acid. These findings definitely show an extrapituitary action of vasopressin.

Early in 1959 our group reported preliminary experiments showing that synthetic vasopressin, when injected into the arterial circuit of the adrenal glands of the hypophysectomized dog, directly stimulates adrenal cortical secretion (10, 11). It is the purpose of this communication to report the results of this work *in extenso*, together with additional experiments designed to elucidate the mechanism of the adrenocorticotropic action of vasopressin.

METHODS

Adult mongrel dogs were anesthetized by the intravenous administration of sodium pentobarbital. When desired, hypophysectomy by the trans-buccal approach of McClean (12) was performed, generally 1–3 days before the day of adrenal perfusion.

In all experiments, the adrenals were perfused using the *in situ* technique of Hilton *et al.* (13). In this preparation arterial heparinized blood obtained from donor dogs is pumped at a constant rate of 10 ml. per minute into the arterial circuit of the isolated adrenal glands of the recipient animal. At the commencement of the pump perfusion the heart of the recipient animal is fibrillated, and the glands are thus perfused entirely with donor blood for the duration of the experiment. Side arms of the tubing leading into the arterial circuit of the adrenal glands allow for continuous blood pressure recording within the isolated adrenal pouch preparation as well as for the injection or infusion of various agents (13).

The venous outflow from the adrenal veins was collected in iced graduated cylinders. In none of the experiments was any of the venous effluent from the adrenals allowed to recirculate or return to the recipient's general circulation. Hydrocortisone secretion was calculated from the measurement of the venous blood flow and the arteriovenous difference of hydrocortisone concentration.

After several control collections, the agents[5] under study for their adrenocorticotropic activity were infused via a side arm in the arterial perfusing circuit and additional collections were obtained. All substances were dissolved or diluted in normal saline before infusion into the glands. They were given at a rate of 1 ml./min. in every instance.

At the end of each experiment, an injection of ACTH (Upjohn Corticotropin Injection, commercial preparation) was given to verify adrenal viability and to compare the hydrocortisone secretion rates with the previous infusion periods.

It should be emphasized that in this type of preparation the perfusing blood and infused materials circulate only once through the adrenals. There is no recirculation, and the glands are not under the influence of other endocrine glands or organs since they are perfused directly via their isolated arterial connections. In most experiments, previously hypophysectomized donor and recipient animals were used so that there was no endogenous ACTH present. Under these conditions, control hydrocortisone secretion rates from the glands were low.

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[5] Professor Vincent du Vigneaud's laboratory most kindly supplied the synthetic lysine, arginine, and acetyl arginine vasopressins as well as the synthetic oxytocin used. Dr. Mary A. Root of the Lilly Research Laboratories generously donated crystalline glucagon.

TABLE 1. INCREMENT IN HYDROCORTISONE (ΔF) SECRETION FROM ADRENAL VEIN FOLLOWING ADMINISTRATION OF LYSINE VASOPRESSIN AS COMPARED WITH THAT FOLLOWING ACTH

Expt. no.	before ACTH F μg/min.	Lysine Vasopressin dose	duration	ΔF μg/min.	after ACTH[1] ΔF μg/min.	Vasopressin[2] responsiveness
			HYPOPHYSECTOMIZED			
1.	0.3	0.22 u/min.	10 min.	0.7	2.0	35%
2.	0.3	0.3 u/min.	7 min.	1.6	3.5	46%
3.	0.2	0.3 u/min.	7 min.	3.3	11.0	30%
			NON-HYPOPHYSECTOMIZED			
4.	2.6	0.22 u/min.	10 min.	6.2	8.1	77%
5.	9.2	0.16 u/min.	7 min.	4.0	2.3	174%
6.	6.0	0.2 u/min.	7 min.	2.4	1.9	126%

[1] One unit per minute for seven minutes

[2] $\frac{\Delta F \text{ after vasopressin}}{\Delta F \text{ after ACTH}} \times 100$

Hydrocortisone concentrations were determined by the method of Peterson, *et al.* (14). Spectrophotometric analysis of blood samples was performed using a Beckman DU spectrophotometer and compared to that of samples of pure hydrocortisone alcohol.[6] Aldosterone concentration in the adrenal venous blood was measured by a double isotope dilution technique.[7] Methylene chloride extracts of plasma were subjected to descending paper partition chromatography using the Bush B_4 system (15), and the resultant chromatographic pattern was compared to that of known reference standards.

RESULTS

Experiments with synthetic lysine vasopressin

Six experiments were performed and are summarized in Table 1. Because of the variability in responsiveness of the adrenals to the standard dose of ACTH from one dog to the next, we have tabulated the results in terms of the increment (ΔF) in cortisol secretion occurring after ACTH. This increment in cortisol secretion above the control secretion rate gives a measure of the maximal responsiveness or sensitivity of the glands in each dog to this supramaximal dose of ACTH of 1 unit per minute for seven minutes[8]

[6] We are grateful to Dr. John M. Chemerdo of Merck, Sharpe & Dohme for a generous supply of this substance.

[7] We are indebted to Dr. James O. Davis of the National Heart Institute for these determinations.

[8] In our preparation hydrocortisone secretion first becomes detectable following 1.0 mU. ACTH and becomes maximal at 10 mU. All injections in this type of assay were given as single doses of 1 ml. each, there being no recirculation of the ACTH through the glands (16).

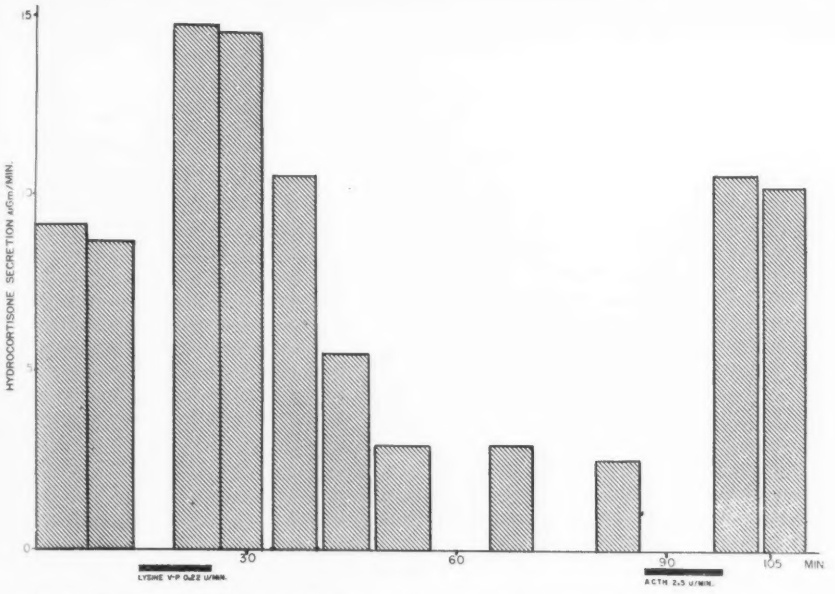

FIG. 1. Experiment showing effect of lysine vasopressin on hydrocortisone secretion. Adrenal glands of non-hypophysectomized dog perfused with blood from a hypophysectomized donor.

The increment in cortisol secretion following vasopressin was then compared with that following ACTH and expressed as a percentage of the ACTH response. As seen in Table 1, the amount of vasopressin perfused into the arterial circuit of the glands varied from 0.16 U./min. to 0.3 U./min. for 7 to 10 minutes. There did not appear to be any correlation between these small differences in dose and the magnitude of stimulatory effect on cortisol secretion. In four of the experiments (Nos. 1–4), the stimulation of cortisol secretion was less than that following ACTH, and in two experiments (Nos. 5 and 6), it was greater than that following administration of ACTH.

Figure 1 depicts the complete protocol of one of these experiments (No. 4, Table 1). In this experiment, a non-hypophysectomized recipient animal was perfused with blood from a hypophysectomized donor. The surgical stress in preparing the adrenal glands for perfusion evoked maximal endogenous ACTH secretion so that even though pump perfusion with ACTH-free blood was begun 25 minutes before zero time seen in Figure 1, the lands were still secreting at a high rate. Cortisol secretion averaged 8.8 µg./min., in the two control periods before vasopressin administration. Following vasopressin there was an increment in secretion of 6.2 µg./min. to a level of 14.8 µg./min. These higher rates of cortisol secretion persisted for 10

minutes after the end of the vasopressin injection. During the next 45 minutes cortisol secretion gradually declined to a low of 2.6 μg./min. under the influence of perfusion with the ACTH-free blood. When exogenous ACTH was given at the end of the experiment, there was an increment in cortisol secretion of 8.1 μg./min. to a level of 10.7 μg./min. Thus, although the total cortisol secretion level was greatest after vasopressin, the increment rise after ACTH indicated greater sensitivity of the glands to ACTH.

In this series of synthetic lysine vasopressin experiments, mean blood

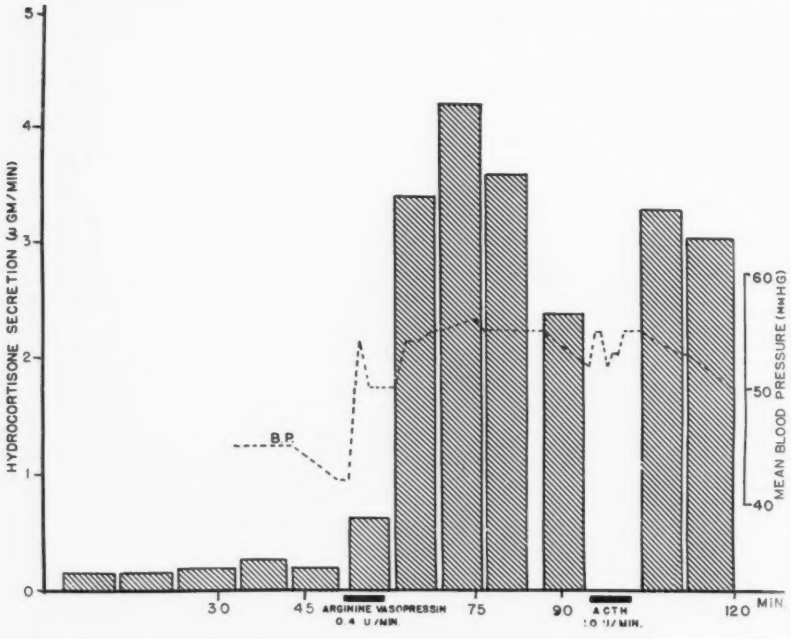

FIG. 2. Effect arginine vasopressin. Adrenal glands of hypophysectomized dog perfused with blood from a hypophysectomized donor.

pressure in the adrenal arterial circuit rose an average of 27 mm. Hg with a range of 8 to 41 mm. Hg during administration of the hormone. There was no pressor activity associated with ACTH administration.

Experiments with synthetic arginine vasopressin

Figure 2 depicts a typical example of this group of experiments. Both recipient and donor animals had been completely hypophysectomized and consequently control cortisol secretion rates were very low, averaging less than 0.2 μg./min. Following the injection of arginine vasopressin (0.4 U./min. for 7 minutes) directly into the adrenal arteries, there was marked

stimulation of cortisol secretion to a peak of 4.2 μg./min. representing an increment of 4.0 μg./min. above control. Mean blood pressure rose an average of 10 mm. Hg. Thirty minutes after the end of the vasopressin injection cortisol secretion had fallen to 2.4 μg./min. At this time 1 unit per minute of ACTH was injected for 7 minutes and cortisol secretion rose to 3.3 μg./min.

In an attempt to define a dosage-response relationship, eight additional experiments were performed in completely hypophysectomized animals. These experiments are summarized in Table 2, which is similar in design to Table 1. There is a good correlation between dose of vasopressin and response of the glands.

TABLE 2. ILLUSTRATING EFFECTS OF GRADED DOSES OF ARGININE VASOPRESSIN ON INCREMENT ON HYDROCORTISONE (ΔF) SECRETION IN HYPOPHYSECTOMIZED DOGS

No. expts. performed	before ACTH av. F μg/min.	Arginine Vasopressin dose (u/min.)[1]	Arginine Vasopressin av. ΔF μg/min.	after ACTH[2] av. ΔF μg/min.	Vasopressin[3] responsiveness
2	< 0.1	0.001	0.4	3.1	13%
2	< 0.1	0.01	1.6	4.1	39%
3	< 0.1	0.1	2.5	3.6	69%
2	0.15	0.4	4.5	3.8	118%

[1] All doses given over 7 minute period

[2] One unit per minute for 7 minutes

[3] $\frac{\text{av. } \Delta F \text{ after vasopressin}}{\text{av. } \Delta F \text{ after ACTH}} \times 100$

In two additional experiments non-hypophysectomized animals were used. Both experiments showed high rates of cortisol secretion in control periods (7 μg./min. and 2.1 μg./min. respectively), and in both experiments cortisol secretion increased following the injection of arginine vasopressin (to 12 μg./min. and 4.3 μg./min. respectively).

Since in all experiments thus far the vasopressin had been injected only over a seven minute period and since the vasopressin stimulatory effect had more or less disappeared within 20 minutes after the end of the injection, it was thought of interest to determine the effect of a prolonged infusion of arginine vasopressin on cortisol secretion. In Figure 3 such an experiment is shown performed in a hypophysectomized preparation. Despite continuation of the vasopressin infusion (10 mU./min.) for 30 minutes, the cortisol secretion began to fall back to control levels during the infusion. This is in marked contrast to the prolonged stimulation induced by ACTH injected at the rate of 1 unit per minute for 2 minutes. A similar result was ob-

tained in an experiment using non-hypophysectomized animals, i.e., the vasopressin stimulatory effect was not sustained by prolonged infusion.

Experiments in which aldosterone secretion was studied

Four experiments were performed and are summarized in Table 3. It should be noted that in three of the experiments there was definite stimulation of aldosterone secretion following ACTH. In Experiment 4 the donor animal was found to have a small remnant of pituitary tissue remaining

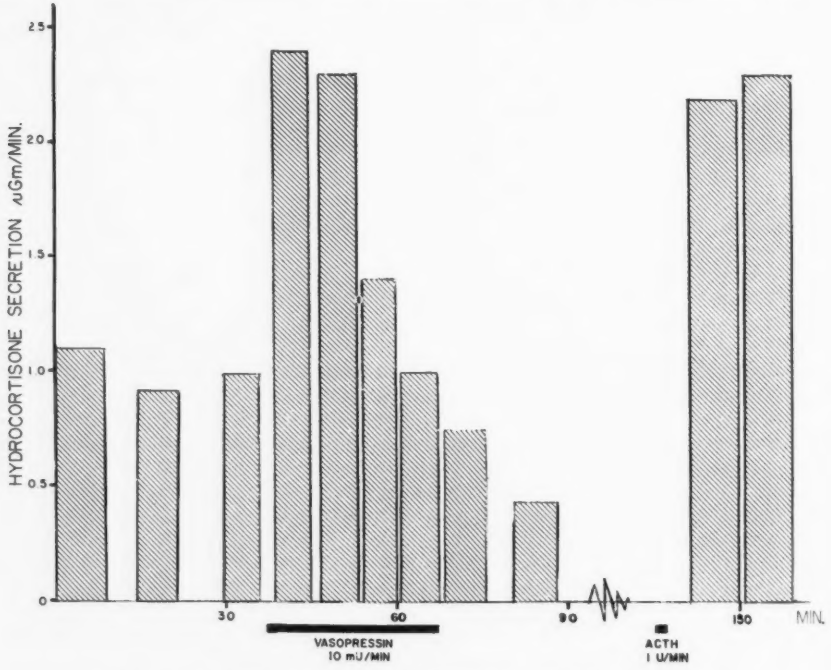

FIG. 3. Effect of thirty minute perfusion of arginine vasopressin on hydrocortisone (F) secretion in hypophysectomized dog.

after bleeding. Apparently this was insufficient to produce maximal cortisol stimulation in the perfused adrenals since there was a further rise after vasopressin and ACTH, but was sufficient to maintain sustained high aldosterone secretion.

Lysine vasopressin (Expts. 1 and 2) although showing its usual stimulatory effect on cortisol secretion, was without effect on aldosterone secretion. On the other hand, acetyl arginine vasopressin showed both hydrocortisone and aldosterone stimulating activity as seen in Experiment 3, Table 3. This compound is synthesized by acetylation of the free amino group of arginine vasopressin, and, on a molecular weight basis, 1.0 microgram of it is ap-

proximately equivalent to 0.4 units of arginine vasopressin. However, in experiments 3 and 4 there was no pressor activity associated with the administration of the acetyl arginine vasopression.

Experiments in which miscellaneous compounds were studied

It was considered of interest to see whether other polypeptides, similar or dissimilar to vasopressin, might have cortisol stimulating activity. In four experiments (two with hypophysectomized and two with non-hypophysectomized preparations), *synthetic oxytocin* in a dosage ranging from 10

TABLE 3. HYDROCORTISONE (F) AND ALDOSTERONE SECRETIONS IN μG./MIN. FOLLOWING INJECTIONS OF VARIOUS SYNTHETIC VASOPRESSINS. ACTH GIVEN AT END OF EACH EXPERIMENT IN DOSAGE OF 1 UNIT/MIN. FOR 7 MINS. ALL ANIMALS HYPOPHYSECTOMIZED EXCEPT AS NOTED

Expt.	Vasopressin dosage over 7 min. period	Control F	Control aldosterone	after Vasopressin F	after Vasopressin aldosterone	after ACTH F	after ACTH aldosterone
1.	lysine 0.3 u/min.	0.35	0	1.9	0	3.8	0.015
2.	lysine 0.3 u/min.	0.6	0.005	3.5	0.008	11.2	0.029
3.	acetyl arginine 14.3 μg/min.	0.6	0.01	6.5	0.045	6.0	0.057
4. *	acetyl arginine 28.6 μg/min.	4.3	0.163	11.0	0.141	10.2	0.132

* Donor animal incompletely hypophysectomized at time of bleeding.

units/min. for one minute to 1.4 units/min. for seven minutes had no activity. At these high doses we did note, however, pressor activity.

In two experiments with hypophysectomized and in three experiments with non-hyophysectomized animals *insulin* in a dosage of 1 unit/min. to 80 units/min. for 1 to 10 minutes had no effect on mean blood pressure or cortisol secretion.

Glucagon in 6 hypophysectomized and 14 non-hypophysectomized preparations had no effect either on cortisol secretion or on mean blood pressure. Dosages used ranged from 0.01 mg./min. to 1.0 mg./min. for 1 to 38 minutes.

Finally, *norepinephrine* (4 μg./min. for 7 minutes) and *epinephrine* (2 μg./min. for 7 minutes) had no effect on cortisol secretion. The former compound did, however, cause a mean blood pressure rise of 14 mm. Hg in the perfused adrenals.

3′,5′ adenosine monophosphate (3′,5′ AMP) has been shown to have potent adrenocorticotropic activity (17, 18). Arginine vasopressin was injected into an adrenal gland preparation being perfused with 3′,5′ AMP at

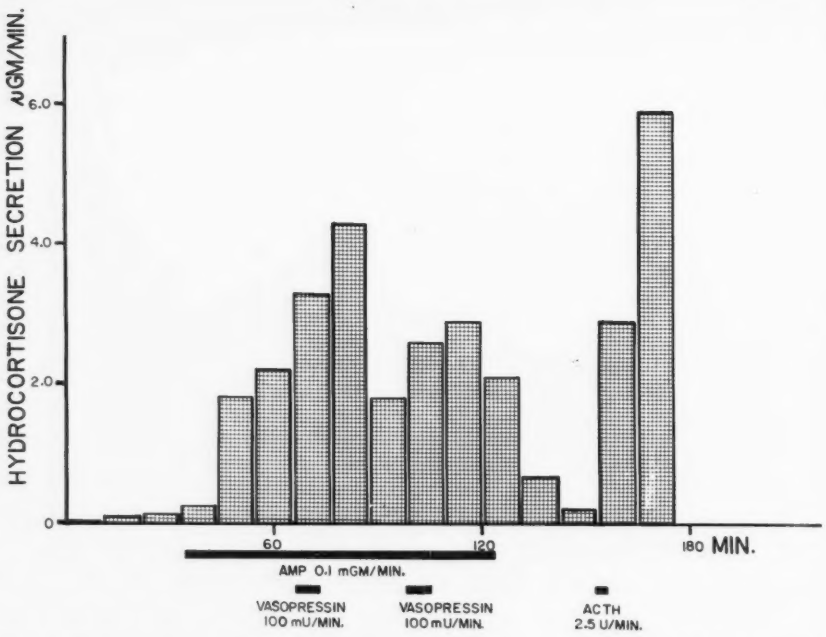

FIG. 4. Hydrocortisone stimulatory activity of 3′,5′ adenosine monophosphate compared with that of arginine vasopressin and ACTH in hypophysectomized dog.

0.1 mg./min. The cortisol stimulatory activity of 3′,5′ AMP is illustrated in Figure 4. Following each injection of arginine vasopressin (100 mU./min. for 7 minutes) there was a transient increment in cortisol secretion of 2.1 μg./min. and 1.1 μg./min. respectively. Immediately upon cessation of the 3′,5′ AMP infusion cortisol secretion fell to basal levels.

DISCUSSION

In 1958 Royce and Sayers (9) demonstrated that vasopressin had an ACTH-like activity in the hypophysectomized rat. They suggested that vasopressin might directly stimulate the adrenal cortex but did not pursue this further. Instead, they proposed that it released ACTH from non-adenohypophyseal binding sites. It would appear that their first suggestion was correct. Our observations clearly demonstrate a direct cortisol stimulating action of synthetic lysine, arginine and acetyl arginine vasopressins when they are perfused through the isolated adrenal glands. That this secretion is hydrocortisone was confirmed by: (1) spectrophotometric analysis of the plasma Porter-Silber chromogens which revealed maximal absorption at 410 mμ, similar to that of hydrocortisone alcohol, and (2) paper chromatography of the plasma methylene chloride extracts which showed a motility identical to hydrocortisone alcohol (RF = 18).

Minimal cortisol stimulatory effects of arginine vasopressin, the naturally occurring form in dog and man, were noted with amounts as little as 0.02 μg. (0.007 units) infused over a 7 minute period (Table 2, 1μg. = 0.4 units). This is to be contrasted with the 2.0 μg. amount of arginine vasopressin needed to show cortisol stimulatory activity *in vitro* (19). Maximal cortisol stimulation occurred in our preparation at arginine vasopressin dosages ranging between 100 and 400 mU./min. for 7 minutes. With these amounts secretion of cortisol was equal to or greater than that seen after supramaximal doses of ACTH. It might be argued that such vasopressin dosages are unphysiological. However, appreciable activity was seen with total dosages of 70 mU. and detectable effects were noted with total dosages of 7 mU. In one experiment, not included in our tabulated data, a definite response was seen following 0.7 mU. This could not be reproduced in additional experiments and we concluded that with such small amounts deterioration of the vasopressin may have occurred[9] or that the adrenal glands of these preparations were less sensitive.

It would appear reasonable to ascribe a role to arginine vasopressin in the "stress reaction." In favor of this hypothesis is the work of McCann, Fruit and Fulford (20) who showed that the antidiuretic action of electric shock in rats could be significantly diminished by pretreatment with hydrocortisone. Similar neurohypophyseal inhibition in Δ^1 hydrocortisone treated animals has been reported by de Wied and Mirsky (21). Conversely, in adrenal insufficiency increased quantities of circulating ADH have been found (22). This would suggest a "feed-back" mechanism between the adrenal cortex and the neurohypophysis. Furthermore, in the stresses associated with acute blood loss (23) and with anesthesia (24) vasopressin levels in the blood increase. Also, Frieden and Keller (25) using diabetes insipidus dogs found them to be more sensitive to the hypotensive effects of hemorrhage than normal animals, indicating loss of some protective mechanism. One might speculate on whether this protective effect is a direct effect of vasopressin or whether this is mediated via the liberation of adrenocortical hormone.

Recently it has been shown that as little as 2 mu. of arginine vasopressin will cause a *maximal* release of ACTH when injected directly into the third ventricle of the dog (26). Since this amount is much less than the 7 mU. needed to stimulate *minimal* cortisol secretion (Table 2) in our preparation, it would appear that arginine vasopressin has ACTH releasing activity as well as a direct cortisol stimulatory activity. Therefore, it is apparent that arginine vasopressin may have three functions in salt and water

[9] Once diluted with saline, vasopressin is very unstable with most of its cortisol stimulatory activity being lost after 2 hours at room temperature. However, pressor activity under these conditions is retained. We have no data regarding stability of antidiuretic activity.

homeostasis: (1) anti-diuretic activity (27), (2) ACTH releasing activity and (3) direct stimulatory activity on the adrenal cortex.

The cortisol stimulatory action of vasopressin on the adrenal cortex was highly specific. It was not seen following administration of other polypeptides such as oxytocin, insulin and glucagon or pressor amines such as norepinephrine and epinephrine. We have not had the opportunity to test the corticotropin releasing factor (CRF) of Schally and Guillemin (28) which does not have cortisol stimulatory activity when tested in *in vitro* incubation systems (19).

Pressor activity in the adrenal arterial circuit of our preparation was seen following administration of both lysine and arginine vasopressins, but not with acetyl arginine vasopressin. This last compound has no pressor activity in the standard rat pressor assay method and also blocks the pressor action of arginine vasopressin in that assay (29). Cortisol stimulating activity of the acetylated derivative was identical to that of the non-acetylated compound in our perfusion preparation. These observations indicate a dissociation between the pressor action and the cortisol stimulatory activity. A similar dissociation in *stability* of these two effects was seen with arginine vasopressin.[9]

In the experiments in which aldosterone secretion was studied (Table 3), ACTH in every instance caused highly significant increases in secretion. This ACTH effect has been observed by others in acute experiments (30, 31) but cannot be reproduced by the chronic administration of ACTH (32). The reasons for this disparity are not clear but may lie in the differences in methods employed—the acute studies having been done directly on adrenal vein blood whereas the chronic studies were done on 24 hour urine collections. This latter technique measures only excreted hormone and gives no direct information on secretion since metabolism and destruction of the aldosterone cannot be assessed.

The influence of the posterior pituitary on aldosterone secretions has not been thoroughly evaluated to date. *In vitro* incubation of rat (33) and beef adrenal slices (34) with crude extracts of posterior pituitary lobes has revealed stimulation of aldosterone, hydrocortisone and corticosterone synthesis. Stimulation of aldosterone secretion was not obtained when commercial pitressin or oxytocin was used (34). The validity of these experiments is suspect because ACTH contamination of the crude extracts of posterior pituitary could not be excluded. Furthermore, when purified products of pitressin or vasopressin are used in such *in vitro* systems, the great instability of these preparations (35) might account for loss of aldosterone and cortisol stimulating activity originally present.

It is impossible for us to draw any definite conclusions regarding the effect of vasopressin on aldosterone secretion from our data. In two experiments lysine vasopressin produced no effect. However, the results after acetyl arginine vasopressin are unclear since there appeared to be some

allotropic effect in one experiment but not in the second (Table 3). In this latter experiment endogenous ACTH was present.

Turning to a consideration of the mechanism of the cortisol stimulatory effects of vasopressin we notice one outstanding difference between it and the action of ACTH. ACTH causes continuous stimulation of hydrocortisone secretion during prolonged perfusion of the adrenal glands (13) whereas vasopressin under the same conditions produces transient stimulation (Fig. 3). It is unlikely that the transient effect of vasopressin is due to the release of small amounts of bound ACTH from the adrenal glands. Although an unsustained response is consistent with single small doses of ACTH (16), the animals in several experiments had been hypophysectomized days before perfusion and it is doubtful that any ACTH remained in the adrenal tissue. In addition, vasopressin often caused greater secretion than supramaximal doses of ACTH. Finally, there is no reason to assume ACTH binding at any site, nor is there experimental evidence indicating such binding.

It is known from the experiments of Haynes (18) that ACTH causes the accumulation of 3′, 5′ adenosine monophosphate (3′,5′ AMP) in beef adrenal cortex incubates and that this in turn stimulates adrenal phosphorylase production. Haynes, Koritz and Peron (17) have also shown that 3′,5′ AMP added to rat adrenals incubated *in vitro* elicits a response in corticosteroid production equal to or greater than that produced by ACTH. This is analogous to the finding of Sutherland and Rall (36) that epinephine and glucagon cause a selective increase in the production of 3′,5′ AMP in liver slices and consequent stimulation of liver phosphorylase activity resulting in glycogenolysis.

When 3′,5′ AMP is perfused through the intact adrenal glands (Fig. 4), cortisol secretion is enhanced in a manner identical to that seen after ACTH and adds support to the theory (17) that corticosteroid synthesis is mediated by stimulation of adrenal phosphorylase production. Since the effect of vasopressin on cortisol secretion is relatively short-lived compared to that of 3′5′ AMP and ACTH, it is conceivable that vasopressin acts in some manner to activate adrenal phosphorylase rather than actually stimulating its production. It is of interest that posterior pituitary extract has been found to show liver glycogen depleting ability (37). Recently it has been demonstrated that synthetic lysine vasopressin causes increased secretion of glucose in the perfused dog liver preparation in a manner similar to that seen after glucagon (38). This would suggest that vasopressin can stimulate liver phosphorylase as well as adrenal phosphorylase.

REFERENCES

1. Mirsky, I. A., M. Stein and G. Paulisch: *Endocrinology* **54**: 491. 1954.
2. Fraja, A. and L. Martini: *Arch. Int. Pharmacodyn.* **93**: 167. 1953.
3. McCann, S. M. and A. Fruit: *Proc. Soc. Exper. Biol. and Med.* **96**: 566. 1957.

4. McCann, S. M.: *Endocrinology* **60:** 664. 1957.
5. McCann, S. M., and J. Brobeck: *Proc. Soc. Exper. Biol. and Med.* **87:** 318. 1954.
6. McDonald, R. K. and K. Weise: *Proc. Soc. Exper. Biol. and Med.* **92:** 107. 1956.
7. McDonald, R. K. and K. Weise: *Proc. Soc. Exper. Biol. and Med.* **92:** 481. 1956.
8. Hume, D. M. and D. Nelson: The Endocrine Society (abstract). P. 98. 1957.
9. Royce, P. C. and G. Sayres: *Proc. Soc. Exp. Biol. and Med.* **98:** 70. 1958.
10. Hilton, J. G., L. Scian, C. Westermann and O. Kruesi: *Proc. Soc. Exper. Biol. and Med.* **100:** 523. 1959.
11. Hilton, J. G., L. Scian, C. Westermann and O. Kruesi: *Science* **129:** 971. 1959.
12. McClean, A. J.: *Ann. Surg.* **88:** 985. 1928.
13. Hilton, J. G., D. Weaver, G. Muelheims, V. Glaviano and R. Wegria: *Am. J. Physiol.* **192:** 525. 1958.
14. Peterson, R. E., A. Karrer and S. L. Guerra: *Analytical Chem.* **29:** 144. 1957.
15. Bush, I. E.: *Biochem. J.* **50:** 370. 1952.
16. Hilton, J. G.: Unpublished Observations.
17. Haynes, R. C., Jr., S. Koritz and F. Peron: *J. Biol. Chem.* **234:** 1421. 1959.
18. Haynes, R. C., Jr.: *J. Biol. Chem.* **233:** 1220. 1958.
19. Guillemin, R. and A. Schally: *Endocrinology* **65:** 555. 1959.
20. McCann, S. M., A. Fruit and B. Fulford: *Endocrinology* **63:** 29. 1958.
21. de Wied, D. and I. Mirsky: *Endocrinology* **64:** 955. 1959.
22. Birnie, J. H., W. Eversole, W. Boss, C. Osborn and R. Gaunt: *Endocrinology* **47:** 1. 1950.
23. Ginsburg, M. and L. Brown: in "The Neurohypophysis" edited by H. Heller, Butterworths Scientific Publications, London, p. 109. 1957.
24. De Bodo, R. C. and K. Prescott: *J. Pharmacol.* **85:** 222. 1945.
25. Frieden, J. and A. Keller: *Circulation Res.* **2:** 214. 1954.
26. Kwaan, H. C. and H. Bartelstone: *The Pharmacologist* **1:** 81. 1959.
27. Orloff, J., H. Wagner, Jr. and D. Davidson: *J. Clin. Investigation* **37:** 458. 1958.
28. Schally, A. V. and R. Guillemin: *Proc. Soc. Exper. Biol. and Med.* **100:** 138. 1959.
29. Studer, R. O., W. Cash and V. de Vigneaud: *J. Biol. Chem.* in preparation.
30. Farrell, G. L., E. Rauschkolb and P. Royce: *Am. J. Physiol.* **182:** 269. 1955.
31. Davis, J. O., R. Bahn, N. Yankopoulos, B. Kliman and R. Peterson: *Am. J. Physiol.* **197:** 380. 1959.
32. Liddle, G. W., L. Duncan, Jr. and F. Bartter: *Am. J. Med.* **21:** 380. 1956.
33. Mialhe-Voloss, C. and E. Baulieu: *Compt. Rend. Acad. Sci.* **246:** 639. 1958.
34. Giroud, C., J. Stachenko and P. Pilletta: in "Aldosterone" edited by A. Muller and C. O'Connor; Little, Brown and Company, Boston, P. 56. 1958.
35. Ressler, C. and J. Rachele: *Proc. Soc. Exper. Biol. and Med.* **98:** 170. 1958.
36. Sutherland, E. W., and T. Rall: *J. Am. Chem. Soc.* **79:** 3608. 1957.
37. Gomori, P. and E. Csomay: *Arch. für Exper. Path. und Pharm.* **175:** 17. 1934.
38. Bergen, S. S., R. Sullivan, and J. Hilton: *Fed. Proc.* **19:** 81. 1960.

OBSERVATIONS ON SERUM AND GONAD CHOLESTEROL IN PIGEONS

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ABSTRACT

Serum cholesterol of pigeons undergoes a seasonal cycle in concentration with maximal values present in April and minimal values in August. No sex differences were apparent and both sexes exhibited this yearly fluctuation in cholesterol concentration. These seasonal changes occurred even when only males showing histological evidence of spermatogenesis were considered. The suggestion is made that perhaps serum cholesterol is not regulated by the sex steroids in this species.

Cholesterol concentration is high in the small undeveloped testis and declines rapidly when gonad development starts. This concentration is inversely proportional to the logarithm of relative testis weight. The logarithm of cholesterol content of the testis is directly related to the logarithm of testis weight. The data suggest that testicular cholesterol is involved in gonad growth and increased mass.

THE functional significance of cholesterol in male gonads has never been clearly elucidated. Several investigators have demonstrated, by histochemical means, that cholesterol is usually localized in the interstitial cells and have suggested that this compound might function as a precursor for androgenic hormones (1, 2). Recently however, Pearlman (3, 4) showed, with refined histochemical and chemical techniques, that the amount of cholesterol in the interstitial cells of rat testis was small compared to that found in the tubules and suggested that this compound was probably more intimately associated with spermatogenesis. Information regarding the influence of gonadal hormones on serum cholesterol has likewise been controversial (5).

Not much is known concerning gonad or serum cholesterol in the bird or the factors affecting it. General agreement exists that, under most conditions, estrogens tend to increase avian serum cholesterol (6, 7, 8), while androgens either have no effect (7, 8), or protect the bird against experimentally-induced hypercholesteremia (9).

Since birds are primarily seasonal breeders, it appeared logical that serum cholesterol would show considerable yearly fluctuations in relation to changes in sexual activity and likewise show a considerable difference be-

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tween sexes such as is found in rats (5, 10). Additional insight into the functional significance of gonad cholesterol might be obtained by measuring this organic constituent in testes showing different stages of sexual development and regression.

This report presents data concerning seasonal changes in serum and gonad cholesterol for both male and female pigeons. While this species should not be strictly considered as a seasonal breeder, individuals do undergo a yearly cycle of sexual development and regression.

MATERIALS AND METHODS

A total of 220 pigeons supplied the data for this report. At least 14 birds were sacrificed during each month. The birds were obtained from local suppliers and held for variable lengths of time in a large outdoor enclosure with access to an indoor loft. No special diet or feeding regimen was employed.

Blood was withdrawn from the wing veins and serum cholesterol was measured colorimetrically according to the method of Zlatkis *et al.* (11). The following day, the birds were killed with ether and the gonads removed and weighed individually to the nearest milligram. The ovaries of the females were not weighed but, rather, relative development was determined by measuring the largest diameter of the largest follicle. Gonad cholesterol was determined by the procedure described by Knobil *et al.* (12) for analysis of the adrenal gland.

Mean gonad and serum cholesterol values were obtained for each month and sex, and standard errors of the means were calculated by the method of Tippet (13). Samples of all testes were fixed, sectioned and stained for microscopic examination of the stage of spermatogenic activity.

RESULTS

A wide variation between individual serum cholesterol values appears to be characteristic of the pigeon and consequently large standard errors of the means are the rule.

Serum cholesterol of both the male and female pigeon showed a characteristic seasonal fluctuation in amount. Maximal values were found in the spring months of March, April and May and minimal values were obtained in July and August (Table 1). Interestingly enough, no significant difference in serum cholesterol was apparent between males and females. Consequently, when the values of both sexes were combined and plotted, maximal serum concentration occurred in April and the minimum in August (Fig. 1).

It is pertinent that active spermatogenesis was found in some birds in all months of the year except during December when spermatids were found to be the most advanced stage of development. On the other hand, all males examined in March and April showed active spermatogenesis with abundant sperm in the tubules. The presence of sexually active males in all or most of the months is not surprising since some fertile eggs have been found in Baltimore nesting sites throughout the year (14).

No correlation was found between gonad development or weight and

TABLE 1. MONTHLY CHANGES IN SERUM CHOLESTEROL CONCENTRATION OF MALE AND FEMALE PIGEONS

Month	Males, No. birds	Cholesterol (mg./100 ml. serum ±S.E.)	Females, No. birds	Cholesterol (mg./100 ml. serum ±S.E.)
Oct.	11	242 ± 17	4	248 ± 17
Nov.	14	292 ± 8	5	253 ± 17
Dec.	8	323 ± 21	12	314 ± 22
Jan.	18	312 ± 11	7	312 ± 18
Feb.	17	407 ± 22	9	403 ± 34
Mar.	16	399 ± 14	5	461 ± 44
Apr.	8	492 ± 62	9	455 ± 50
May	9	418 ± 29	5	455 ± 39
June	12	359 ± 12	4	355 ± 29
July	7	217 ± 10	9	227 ± 9
Aug.	8	220 ± 11	7	182 ± 15
Sep.	8	267 ± 8	8	261 ± 7
Total	136		84	
Mean		329		327

serum cholesterol. All birds showing histological evidence of spermatogenesis presented as much variation in serum cholesterol concentration as those showing sexual inactivity. Both groups also showed similar seasonal changes in serum concentration. Likewise, within single months or groups of months, no significant difference was found in serum concentration between females having ovaries with follicles under 2.0 mm., over 4.0 mm. or between.

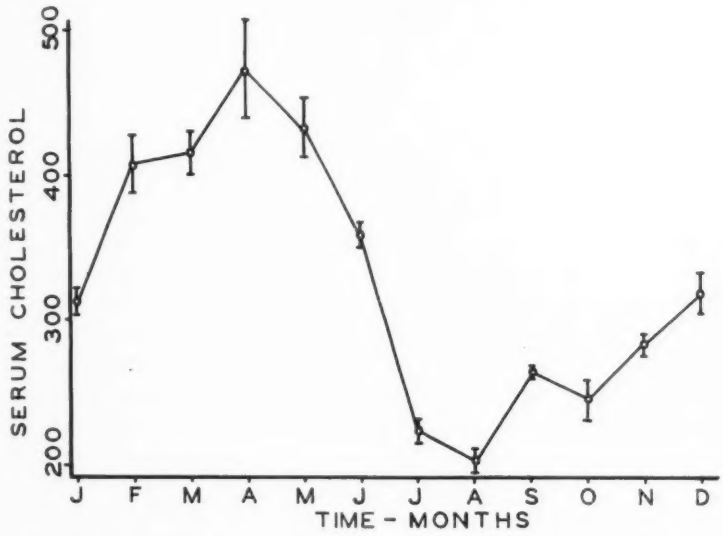

FIG. 1. Monthly changes in serum cholesterol of male and female pigeons. Mean ±S.E.

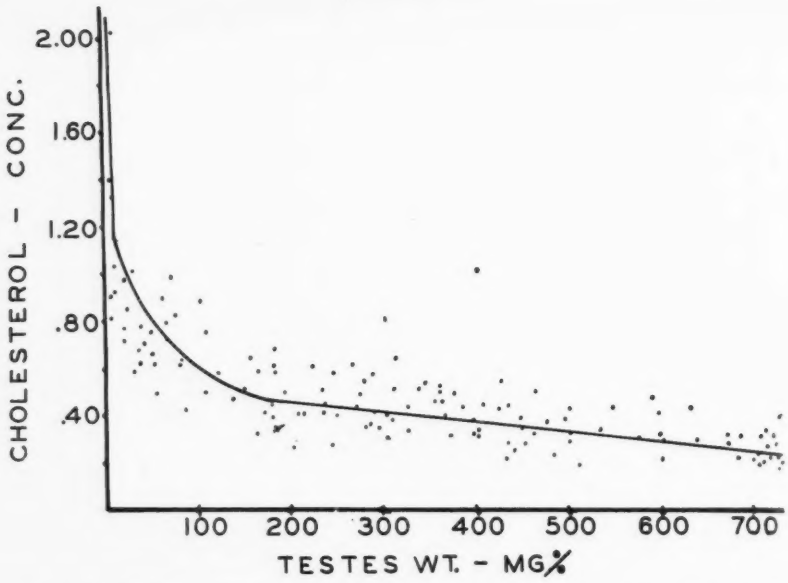

FIG. 2. Cholesterol concentration versus relative testis weight. Curve fitted by inspection.

When the concentration of testis cholesterol is plotted as a function of relative testis weight (Fig. 2), highest values are found in the extremely small undeveloped gonads. As sexual stimulation commences however, this high concentration immediately falls at a rate out of proportion to the increase in weight or spermatogenic activity. At a testis weight of approximately 100 mg./100 gm. body weight, this rate of decline in concentration tapers off considerably to a rather flat curve. At weights above 300–400 mg./100 gm. body weight, the concentration varies with mean values of approximately 0.30–0.40 mg.%.

When concentration of cholesterol is plotted as a function of the logarithm of the relative testes weight, a linear relationship is obtained (Fig. 3). It is quite apparent that as testis weight (x) increases logarithmically, the cholesterol concentration (y) declines according to the following relationship: $y = 1.56 - 0.43 \log x$, with a correlation coefficient of $r = -0.75$.

Sperm were found in testes (both) weighing from 621 mg. (142 mg./100 gm. body weight) up to the maximum of 3150 mg. (769 mg./100 gm. body weight); when only these animals showing full spermatogenesis were considered, cholesterol concentration also varied according to the curve in Figure 3.

Total cholesterol (mg.) present in both gonads showed extreme variation at all testes weights (mg.) except in those totalling 200 mg. or under. In this weight range, the variation was slight and the total amount was di-

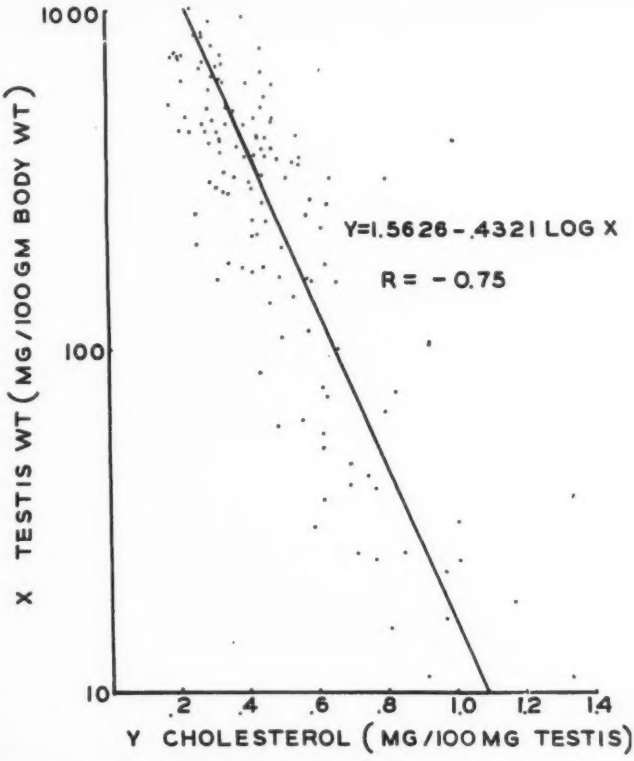

FIG. 3. Cholesterol concentration versus relative testis weight (log.).

rectly related to total weight ranging from approximately 0.25 mg. in testes weighing 30 mg. to 1.5 mg. in testes of 200 mg. Thereafter, the direct relationship did not hold. When, however, the logarithm of total cholesterol is plotted as a function of the logarithm of the total testes weight, a definite linear relationship is apparent (Fig. 4). This relationship exists according to the formula for a straight line calculated to be: Log $y = -1.31 + 0.65$ log x, where y = total amount of cholesterol and x = total testes weight. The correlation coefficient was calculated to be: $r = 0.72$.

No differences were noted between sexually active testes at different seasons of the year. It is interesting that in some birds, the two testes were considerably different in weight, size and histology. However, the concentration and total amount of cholesterol were always found to be related to the testis weight according to the curves of Figures 3 and 4. This would suggest that testis cholesterol is independent of circulating levels of androgenic or pituitary hormones; rather, it changes in relation to growth or development.

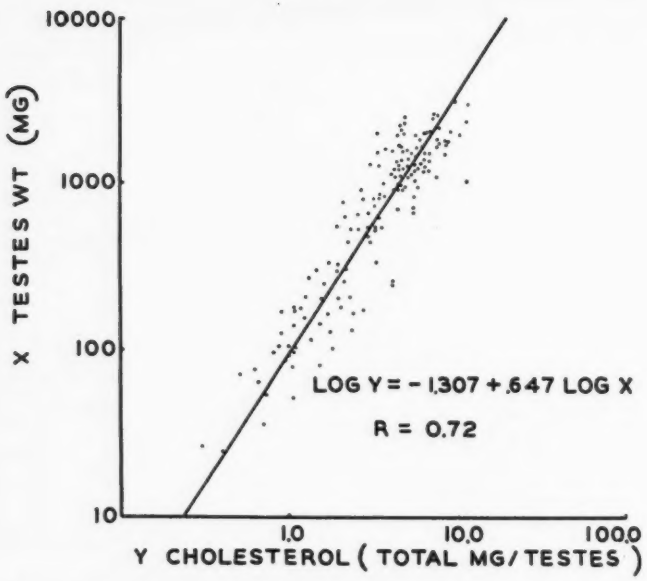

FIG. 4. Total cholesterol content versus total testis weight (Log-Log plot).

DISCUSSION

The regulation of serum cholesterol is, as yet, unknown. A gonadal control has been postulated in birds (6, 7, 8) and in mammals (5, 15), but controversy exists concerning the actual effects of estrogens and androgens. Differences exist, depending on compound, dose, sex, sexual activity and species (16, 17). In view of this uncertainty plus the fact, 1. that both male and female pigeons show similar seasonal changes in serum cholesterol, 2. no sex differences in serum cholesterol were found, contrary to the situation in most mammals including man (5, 10, 18), 3. this seasonal fluctuation was evident even when only males showing histological evidence of active spermatogenesis were considered and 4. the pigeon is not a purely seasonal breeder, permits the tentative conclusion that perhaps serum cholesterol is not normally regulated by the sex steroids in this species.

Other factors which might act to regulate or influence serum cholesterol would include the thyroid gland. It is known that serum cholesterol normally declines following the administration of thyroid hormones and increases in the hypothyroid state (16, 17). The thyroid gland of both mammals and birds also fluctuates in activity throughout the season under changes in environmental temperature and probably other factors (15–25). Information is available that thyroid gland activity of some birds is highest in the summer months in association with parental care and molting and lowest in late winter (26, 27). In chickens, highest activity is found in

October–November and lowest in February–March (22). These periods of lowest activity nearly coincide with the highest serum cholesterol found in this study while lowest cholesterol values occurred in July–August during the normal molting period.

Some workers have demonstrated a depressant effect of estrogens on thyroid activity (28, 29). The question is raised, therefore, whether estrogens produce an indirect increase (6, 7, 8) in serum cholesterol by virtue of thyroid gland involvement. Further experimentation along these lines is necessary. In the absence of experimental data to the contrary, the data from pigeons do not support the concept of gonadal regulation of serum cholesterol. Rather it appears more probable that other factors including the thyroid gland regulate serum cholesterol, modified perhaps by changing levels of gonadal hormones.

Due to the variation in the inactive testis weight, a decline in cholesterol concentration is evident before a real increase in weight or development is discernible. Likewise, the cholesterol content shows a concurrent increase with testicular development. It is apparent that initial stimulation of the male pigeon gonad is immediately followed by a rapid and dramatic change in cholesterol metabolism and mobilization.

Spermatogenesis was found in testes weighing from 600 mg. to over 3000 mg. When only these testes were considered, cholesterol content and concentration continued to change with testes weight as shown in Figures 3 and 4. Thus, testicular cholesterol appears to be primarily involved with testis growth and mass rather than with spermatogenesis. A direct relationship of cholesterol to spermatogenesis seems to exist only in so far as spermatogenesis and growth are inseparable during the initial phases of development.

If cholesterol is primarily involved with growth of the testes, then the apparent contradictions regarding the location and possible significance of cholesterol might be resolved. This organic constituent might be present in varying degrees in both interstitial and spermatogenic cells. The relative presence or absence in each location, depending on the technique and the species or age of the animal, could be due to cyclic waves of growth and increasing tissue mass of these two testicular components. Additional experimentation will be necessary to further elucidate the physiological significance of cholesterol.

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REFERENCES

1. Kuntz, A.: *Arch. f. Mikro. Anat.* **96:** 387. 1922.
2. Pollach, W. F.: *Anat. Rec.* **84:** 23. 1942.
3. Pearlman, P. L.: *Endocrinology* **46:** 341. 1950.

4. Pearlman, P. L.: *Endocrinology* **46:** 347. 1950.
5. Fillios, L. C., R. Kaplan, R. S. Martin and F. J. Stare: *Am. Jour. Physiol.* **193:** 47. 1958.
6. Lorenz, F. W., I. L. Chaikoff and C. Entenman: *J. Biol. Chem.* **126:** 763. 1938.
7. Entenman, C., F. W. Lorenz and I. L. Chaikoff: *J. Biol. Chem.* **134:** 495. 1940.
8. Riddle, O. and T. Senum: *Anat. Rec.* **75:** 58 (abst.), 1939.
9. Cook, D. L., R. A. Edgren and T. W. Harris: *Circ. Res.* **5:** 54. 1957.
10. Fillios, L. C.: *Endocrinology* **60:** 22. 1957.
11. Zlatkis, A., B. Zak and A. J. Boyle: *J. Lab. & Clin. Med.* **41:** 486. 1953.
12. Knobil, E., M. G. Magney, E. J. Wilder and F. N. Briggs: *Proc. Soc. Exp. Biol. and Med.* **87:** 48. 1954.
13. Tippet, L. H. C.: *Biometrika* **17:** 364. 1925.
14. Davis, D. Personal communication 1958.
15. Fillios, L. C. and G. U. Mann: *Circ. Res.* **4:** 406. 1956.
16. Kretchevsky, D.: Cholesterol. John Wiley & Sons, N. Y. 1958.
17. Cook, R. P. Cholesterol; Chemistry, Biochemistry and Pathology. Acad. Press Inc., N. Y. 1958.
18. Katz, L. N. and J. Stamler: Experimental Atherosclerosis: Springfield, Illinois, 1953.
19. Seidell, A. and F. Fenger: *J. Biol. Chem.* **13:** 517. 1913.
20. Adams, A. E.: *Quart. Rev. Biol.* **21:** 1. 1946.
21. Riddle, O. and W. S. Fischer: *Am. Jour. Physiol.* **72:** 464. 1925.
22. Reineke, E. P. and C. W. Turner: *Poult. Sci.*, **24:** 499. 1945.
23. Hoffman, R. A. and M. X. Zarrow: *Acta Endocrinol.*: **27:** 77. 1958.
24. Davis, J. and B. S. Davis: *Condor* **56:** 328. 1954.
25. Kendeigh, S. C.: *Ecol. Mono.* **4:** 299. 1934.
26. Kuchler, W.: *Jour. Ornithol.* **83:** 414. 1935.
27. Elterich, C. F.: *Endokrin. Bd.* **18:** 31. 1936.
28. Farbmann, A. A.: *J. Clin. Endocrinol. and Metab.* **4:** 17. 1944.
29. Koenig, V. L., F. X. Glassner and R. G. Gustavson: *Am. Jour. Physiol.* **144:** 363. 1945.

EFFECT OF ELECTRICAL STIMULATION OF THE LIMBIC SYSTEM ON PITUITARY-ADRENOCORTICAL FUNCTION: POSTERIOR ORBITAL SURFACE

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ABSTRACT

The posterior orbital surface of the morphine pre-treated dog was electrically stimulated for ten seconds under direct vision following craniotomy. Jugular and adrenal venous blood was collected before, during, and after the stimulation to analyze for ACTH and 17-OHCS respectively. A sharp and transient rise of both ACTH and 17-OHCS was observed immediately after the stimulation, invariably accompanied by somatic responses such as elevation of blood pressure, twitching of nose, respiratory standstill, and pupillary dilatation. None of these responses was seen after stimulation of the anterior sylvian gyrus as control, in hypophysectomized ACTH maintained animals, and in sham-operated animals. Increase of blood ACTH in response to posterior orbital surface persisted after bilateral section of splanchnic nerve. The onset of increased adrenocortical activity, however, seemed to be considerably delayed.

INTRODUCTION

THE regulation of pituitary-adrenal function through the central nervous system has been the subject of many studies, most of them dealing with the hypothalamus (1, 2). Concerning the cerebral cortex, however, intimate relationship with pituitary-adrenal system has not been established, although ACTH release in emotional stress makes such association most likely. The posterior orbital surface has been known to play an important role in the autonomic nervous regulation (3, 4, 10). Porter (5) stimulated the posterior orbital surface of monkeys and cats, obtaining an eosinopenic response suggesting ACTH release. Our investigation was conducted in dogs, using more direct approach of determining blood ACTH and 17-OHCS in relation to electric stimulation, with particular emphasis on the pattern, amplitude and duration of pituitary-adrenal response. We have defined "Posterior Orbital Surface" in dog as the prepyriformic area; transitory region between pyriform lobe and olfactory tract, including part of the insula, as shown in Figure 1.

METHODS

Twenty-one adult mongrel dogs of approximately 10 kg. body weight were used. Operations were performed after subcutaneous administration of 0.4 cc. per kg. b.w.

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2% solution of morphine hydrochloride, under local anesthesia with 2% procaine hydrochloride. Intravenous administration of normal saline and preserved dog blood was used to maintain a constant blood pressure level. Following temporal skin incision, the zygomatic process and the articular process of the mandibula were removed, and craniotomy was performed to visualize the posterior orbital surface directly. The right side was selected in 5 and the left in 3 of the 8 experiments on intact animals and the right side was always used in others. The animals were heparinized at the end of the operation. Adrenal venous blood, 5—10 cc., before, during and 5—30 minutes after the stimulation, was obtained from the right adrenal, through a cannula inserted with translumbar and retroperitoneal approach, following the technic of Hume and Nelson (6), except the substitution of the choker with simple ligation. Right jugular venous blood was collected by direct cannulation 40 cc., before, during and 5 minutes after stimulation. Experiments were not started until 1 hour after the operation, and 2—2½ hours after morphine administration. Single stimulation with concentric electrode, S.W.D., 2 volt, 50 c/s, 5 m. sec., was applied for 10 sec. under direct vision. Four transient characteristic re-

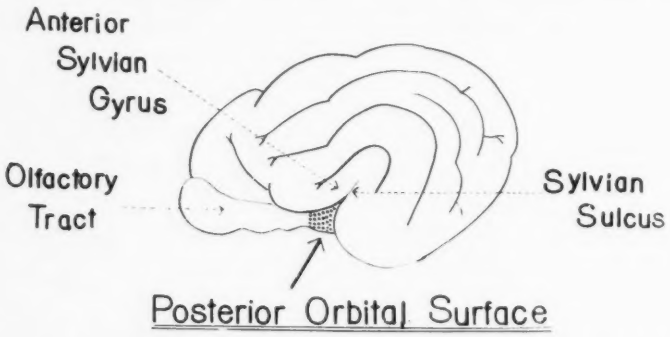

FIG. 1. Diagram of the lateral view of dog brain. The stimulation of the stippled area produced the described effects.

sponses; elevation of blood pressure, twitching of the nose, respiratory arrest, and pupillary dilatation were used as the criteria for successful stimulation. The site was further confirmed in the formalin-fixed brain. For control purposes, anterior sylvian gyrus was stimulated in 5 animals either preceding to or following the stimulation of posterior orbital surface stimulation, and in 2 animals independently. After quickly separating the plasma from the adrenal venous blood, plasma 17-OHCS was determined according to Nelson and Samuels (7). Jugular venous blood was directly collected in 8 cc. of glacial acetic acid and processed according to Fujita (8) to be assayed by the ascorbic acid depletion method in hypophysectomized rats of Sayers, Sayers, and Woodbury (9). One gram oxycellulose was used for every 40 cc. of blood. Hypophysectomy or bilateral section of splanchnic nerves was performed 2 days prior to the experiment. Hypophysectomized dogs were maintained with daily intramuscular injections of 20 units of corticotrophin Z (Organon).

RESULTS

ACTH in jugular venous blood

Before stimulation, ACTH was scarcely detectable in 20 cc. of jugular venous blood.

TABLE 1. BLOOD ACTH ASSAY USING JUGULAR VEIN BLOOD OF DOGS
(Ascorbic Acid Depletion mg./100 gm. by 20 cc. blood)

ntact, Posterior Orbital Surface Stimulation		Mean ± S.E.
Before Stimulation	75, 76, 21, 45, −64, 9, −4, −55	13 ± 19*
During Stimulation	174, 85, 101, 84, 78, 77, 129, 92	103 ± 12*
Five Minutes after Stimulation	85, 114, 40, 97, −13 (*t = 4.00 P < 0.01)	65 ± 23
Splachnic Nerves Sectioned, Posterior Orbital Surface Stimulation		
Before Stimulation	63, 84, −37, 63, 58, 37, 86, 48	50 ± 14*
During Stimulation	168, 80, 78, 80, 195, 175, 109	126 ± 19*
Five Minutes after Stimulation	38, 103, −11, 32 (*t = 3.18 P < 0.01)	41 ± 24
Hypophysectomized, Posterior Orbital Surface Stimulation		
Before Stimulation	−4, −32, −35	−24 ± 10
During Stimulation	23, −95, 11, −35	−24 ± 27
Five Minutes after Stimulation	−32, −37, 9, −14, 1	−15 ± 9
Sham Operated, No Stimulation		
Samples taken at corresponding time I	−37, 54, −57, 71, −36, 16, 33	6 ± 19
II	−46, −65, 31, −67, 14, −105, 52, −4	−27 ± 22
III	11, −16, −1, 25, −21, −65, −40	−15 ± 12
Intact, Anterior Sylvian Gyrus Stimulation		
Before Stimulation	−58, 17, −42, 102, 33	10 ± 29
During Stimulation	52, 4, 41, 31, −67	12 ± 21
Five Minutes after Stimulation	−40, −65, 16, 55, −1, 25	−2 ± 18
Standard ACTH		
2 mu	106, 129, 145, 162, 143	137 ± 9
1 mu	111, 89, 112, 92, 68, 84	92 ± 7
0.5 mu	85, 78, 61, 30, 41, 61, 43	57 ± 8
0.25 mu	39, 90, 16, 59, 25, 36	44 ± 11
Recovered ACTH*		
2 mu	97, 121, 107, 128, 143, 165	127 ± 10
0.5 mu	50, 20, 55, 39, 86, 92	57 ± 11
Recovery	84.2 ± 14.8%	
Blood Blank	16, 21, 6, −5, −8	6 ± 6
Normal Saline	−19, 14, 21, −10, −7, 0, −1	0 ± 5

* Known amount of ACTH was added to hypophysectomized dog blood.

Ascorbic acid depletion by a sample corresponding to 20 cc. blood, however, rose sharply during the stimulation ($P < 0.01$), indicating a detectable amount of ACTH regardless of the side of stimulation (Table 1). Bilateral section of the splanchnic nerve did not abolish such response, ($P < 0.01$) (Table 1) although hypophysectomy completely terminated it, while preserving all somatic responses (Table 1). Sham operation and the stimulation of anterior sylvian gyrus did not evoke a positive response (Table 1).

17-OHCS in adrenal venous blood

In 8 experiments, immediate increase of 17-OHCS was observed upon stimulation, reaching the peak of 215 ± 42% (S.E.) of the prestimulation level 5 minutes later, and subsequently decreased gradually to return the

TABLE 2. CHANGES IN 17-OH-CS (μG./100 CC. ADRENAL VENOUS BLOOD)

	Exp. No.	Before stimulation	During stimulation	After stimulation			
				5 min.	10 min.	20 min.	30 min.
Intact, posterior orbital surface stimulation	1	260	510				
	2	260	500	460	360	260	260
	3	120	230	184	184	144	160
	4	60	116	260	88	70	78
	5	182	292	396	238	250	270
	6	208	384	384	510	214	130
	7	210	410	159	92	76	76
	8	206	433	546	201	156	
Splanchnic nerves sectioned, posterior orbital surface stimulation	1	118	160	278	66	162	
	2	254	317	475	503	274	453
	3	174	180	270	88	216	208
	4	104	145	170		144	
	5	138	195	225	88	123	94
Hypophysectomized, posterior orbital surface stimulation	1	108	90	102	70	70	60
	2	129	102	128	133	129	
	3	90	75	50	48	48	23
Sham operated, no stimulation	1	153	115	138	153	151	
	2	137	115	130	107	119	152
	3	131	135	128	140	126	
	4	112	87	104	142	118	118
	5	127	112	107	132	122	118
Intact, anterior sylvian gyrus stimulation	1	98	96	96	116		
	2	160	160	130		90	
	3	270	222	234	258	200	306
	4	118	146	128	150		
	5	60	54	50	66	56	68
	6	118	98	132	120		138
	7	118	119	131	124	130	150

pre-stimulation level 20 minutes later (Tables 2 and 3). No such response was noted, in 5 sham operated animals, upon stimulation of the anterior sylvian gyrus, or in 3 animals two days after hypophysectomy maintained by ACTH (Tables 2 and 3). In one experiment after hypophysectomy without ACTH, 17-OHCS was scarcely detectable throughout the experiment. In 5 dogs after bilateral section of splanchnic nerve, increase of 17-OHCS occurred in normal amplitude but with considerable delay of onset (Tables 2 and 3). Adrenal venous blood flow did not change remarkably during the experiments, resulting in a close parallelism between 17-OHCS concentration and output.

DISCUSSION

Since the posterior orbital surface in dogs has not been well defined as yet, direct comparison of the above results with those of Porter (5) might not be justified at this stage. Babkin's posterior orbital surface was apparently different from ours (10). Our data, however, definitely indicate an intimate functional relationship between this part of the cerebral cortex and pituitary-adrenal system. Apparent persistence of the reaction after

bilateral section of splanchnic nerve ruled out the possibility of participation of adrenal medulla in ACTH release in this type of experiment, although a delayed response might suggest certain change in adrenal responsiveness.

Peripheral blood ACTH in normal dog during stress was reported as 7.4 ±4.5 mu/100 cc. plasma by Hume (11), using Hume and Nelson's method (12). In our experiments, ascorbic acid depletion by 20 cc. jugular vein blood upon posterior orbital surface stimulation was 103 ±12 mg./100 gm., while AAD by 1 mu ACTH, 93 ±mg./100 gm. and by 2 mu ACTH, 136 ±9 mg./100 gm. In our experiments, increase of blood ACTH was quite prompt upon stimulation, returning almost to the pre-stimulation level 5 minutes later. The change of 17-OHCS followed immediately. Thus it appears quite likely that electric stimulation of the orbital surface gave rise to an instantaneous and transient ACTH release by some unknown mechanisms, most probably via hypothalamus. Release of ACTH has been known to occur quite promptly upon stressful stimuli. After exposure to ether for 45 seconds, the blood ACTH of rat rose 2 minutes later and again decreased after 6 minutes (13). Fortier reported changes of corticosterone content in rat blood after single electroshock, giving similar pattern (14).

TABLE 3. CHANGES IN 17-OH-CS CONCENTRATION IN ADRENAL VENOUS BLOOD EXPRESSED AS PERCENTAGE OF THE PRE-STIMULATION LEVEL

	Exp. No.	Before stimulation	During stimulation	After stimulation			
				5 min.	10 min.	20 min.	30 min.
Intact, posterior orbital surface stimulation	1	100	196				
	2	100	192	177	139	100	100
	3	100	192	153	153	120	133
	4	100	193	433	147	117	130
	5	100	160	218	131	137	148
	6	100	185	185	245	103	63
	7	100	195	76	44	36	36
	8	100	210	265	98	76	
	Mean ±S.E.	100	190 ±5	215 ±42	137 ±23	98 ±13	102 ±18
Splanchnic nerves sectioned, posterior orbital surface stimulation	1	100	136	236	56	137	
	2	100	125	187	198	108	178
	3	100	103	155	51	124	120
	4	100	139	163		138	
	5	100	141	163	64	89	68
	Mean ±S.E.	100	129 ±7	181 ±15	92 ±35	119 ±29	122 ±32
Hypophysectomized, posterior orbital surface stimulation	1	100	83	94	65	65	56
	2	100	79	99	103	100	
	3	100	83	56	53	53	26
	Mean ±S.E.	100	82 ±1	83 ±14	74 ±17	73 ±14	41 ±15
Sham operated, no stimulation	1	100	75	90	100	99	
	2	100	84	95	78	87	111
	3	100	103	98	107	96	
	4	100	78	93	127	105	105
	5	100	88	84	104	96	93
	Mean ±S.E.	100	86 ±5	92 ±2	103 ±8	97 ±3	103 ±5
Intact, anterior sylvian gyrus stimulation	1	100	98	98	118		
	2	100	100	81		56	
	3	100	82	87	96	74	113
	4	100	124	108	127		
	5	100	90	83	110	93	113
	6	100	83	112	102		117
	7	100	101	111	105	110	127
	Mean ±S.E.	100	97 ±5	97 ±5	110 ±5	83 ±12	118 ±3

Stimulation of posterior orbital surface appears so consistent in releasing the pituitary ACTH *in vivo* that it should be useful in studying the physiology of the pituitary-adrenal system. The role of the limbic system other than the posterior orbital surface such as the amygdaloid nuclear complex and hippocampal formation in regulating pituitary adrenal function as higher integrator of hypothalamus was recently investigated by Mason (17, 18) and ourselves (19).

Finally, the use of morphine appears quite important in our experiment. The effect of morphine in blocking the stress and in releasing ACTH has been demonstrated (15, 16). In our experiments conducted more than 2 hours after morphine administration, the ACTH releasing action had already disappeared as shown by the results in sham-operated animals.

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REFERENCES

1. Sayers, G., E. S. Redgate and P. C. Royce: *Ann. Rev. Physiol.* **20:** 243. 1958.
2. Harris, G. W.: Neural Regulation of the Pituitary Gland. Edward Arnold, 1955.
3. Okinaka, S., K. Nakao, H. Nakamura, M. Ikeda, T. Tsubaki, Y. Kuroiwa and Y. Toyokura: *Folia Psychiatr. Neurol. Japon.* **7:** 141. 1953.
4. Bailey, P., and W. H. Sweet: *J. Neurophysiology* **3:** 276. 1940.
5. Porter, R. W.: *Rec. Prog. Horm. Research* **10:** 1, 1954.
6. Hume, D. M.: *Surgical Forum* **5:** 568, 1955.
7. Nelson, D. H., and L. T. Samuels: *J. Clin. Endocrinol. & Metab.* **12:** 519, 1952.
8. Fujita, T.: *J. Clin. Endocrinol. & Metab.* **17:** 512, 1957.
9. Sayers, M. A., G. Sayers, and L. A. Woodbury: *Endocrinology* **42:** 379, 1948.
10. Babkin, B. P., and J. J. Speakman: *J. Neurophysiology* **13:** 55, 1950.
11. Hume, D. M.: Reticular Formation of the Brain: J. Churchill, p. 231, 1958.
12. Nelson, D. H., and D. M. Hume: *Endocrinology* **57:** 184, 1955.
13. Sydnor, K. L., G. Sayers, L. Burgess and L. Heiselt: *Endocrinology* **55:** 621, 1954.
14. Fortier, C., *Acta endocrinol.* **30:** 219, 1959.
15. Briggs, F. N. and P. L. Munson: *Endocrinology* **57:** 205, 1955.
16. Suzuki, T., K. Yamashita, S. Zinnouchi and T. Mitamura: *Nature* **183:** 825, 1959.
17. Mason, J. W.: *Am. J. Physiol.* **196:** 44, 1959.
18. Mason, J. W.: Reticular Formation of the Brain: J. Churchill, p. 645, 1958.
19. Okinaka, S., H. Ibayashi, K. Motohashi, T. Fujita, S. Yoshida and N. Ohsawa: Transactions of the First Asia and Oceania Regional Congress of Endocrinology. p. 39, 1959.

EFFECT OF THYROTROPIN UPON THE INTRATHYROIDAL METABOLISM OF THIOCYANATE-S^{35} [1]

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ABSTRACT

S^{35}-labeled thiocyanate was administered to guinea pigs which had been previously treated with thyrotropin. The TSH pretreated animals exhibited T/S gradients of radioactivity which were twice as great as those of the control animals. Paper chromatography of the thyroid homogenates revealed that sulfate-S^{35} was the chief sulfur product of thiocyante-S^{35} metabolism. Thyrotropin produced an increase in the intrathyroidal concentration of sulfate-S^{35} as compared with the concentration in the controls receiving only labeled thiocyanate. Pretreatment with thiourea abolished the TSH response but augmented the concentration of thiocyanate-S^{35} within the thyroid gland. These results suggest that TSH enhanced the intrathyroidal metabolism of thiocyanate-S^{35}.

THE actions exerted by the thiocyanate and the iodide ions upon the thyroid gland are not wholly independent of one another. In pharmacologic concentrations each is capable of preventing organification of iodide by the thyroid gland (1, 2). Under appropriate conditions each may be goitrogenic (3, 4), but the thiocyanate-induced goiters in rats may be abolished by an iodide excess (5). One of the more striking differences between the two ions is the failure of thiocyanate to be concentrated by either the normal or the iodide-deficient thyroid gland (6). It has been shown in humans that inhibition of the thyroid iodide concentrating mechanism by thiocyanate may be partially eliminated by the administration of thyrotropin (7). Thus, a study has been undertaken to determine the effect of thyrotropin upon the intrathyroidal metabolism of labeled thiocyanate.

MATERIALS AND METHODS

Separate experiments were performed on different days using groups of 6 to 12 guinea pigs[3] of both sexes in each experiment. Although initially the body weights were approximately the same (250–300 gm.), some of the TSH-treated animals suffered moderate weight loss. The animals were caged under identical conditions, receiving Rockland

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[3] Obtained from Samuel E. Rice, Saugus, Mass.

guinea pig diet, vegetables, and water *ad lib.* Half of the animals in each group, selected at random, served as controls.

Potassium thiocyanate-S^{35} [4] was diluted with normal saline so that 0.1 ml. contained 20–50 μc. of the labeled material together with 0.25–0.50 mg. of the stable thiocyanate. Sodium sulfate-S^{35} was also diluted in normal saline to a final concentration which was identical to that of the thiocyanate. TSH (thyrotropin)[5] was dissolved in saline (10.0 U.S.P. units per ml.) and given 24 and 3 hours before the administration of the radioactive materials. All injections were made intraperitoneally, and the dose of TSH was either 2 or 4 units per injection. An equal volume of normal saline without TSH was administered to the controls at the same time. Throughout the experiments most of the animals received approximately 50 μc. of the labeled thiocyanate[6] and 0.2 mg. to 0.5 mg. of the stable. (Sulfate-S^{35} was administered in equivalent doses). On two different occasions groups of animals were given 50 mg. of thiourea 3 hours before the labeled thiocyanate was administered. Starting 2 hours after the injections of the labeled thiocyanate (and sulfate), blood was collected under light ether anesthesia by cardiac puncture with a heparinized syringe after which the animals were sacrified by exsanguination or ether anesthesia. The thyroid glands were removed, cleaned, weighed (wet) on a Roller-Smith torsion balance, dropped into 2 ml. of 10% TCA (trichloroacetic acid) and homogenized by hand in a glass tissue grinder (approximately 4 hours elapsed from first to last animal). After standing overnight the homogenate was centrifuged (2000 rpm) and the supernatant transferred to a graduated cylinder. The precipitate was washed 3–4 times with 2 ml. volumes of distilled water using a glass stirring rod to pulverize the precipitate during each washing. The water was decanted after each centrifugation and combined with the TCA supernatant. Following the final washing, the precipitate was dissolved in 2–3 ml. of 3N NaOH.

Two ml. of 10% cold TCA were blown into 1 ml. aliquots of the guinea pig plasma contained in centrifuge tubes. After the finely dispersed precipitate had settled, the tubes were centrifuged and set aside until the supernatant was assayed for its radioactivity content. Individual standards were prepared *in vitro* by the addition of measured concentrations of thiocyanate-S^{35} to appropriate aliquots of normal guinea pig plasma and thyroid homogenate. The radioactivity in the thyroid precipitates and supernatants, as well as in the plasma supernatants, was measured in a Tri-Carb Liquid Scintillator.[7]

One ml. aliquots of the samples were diluted up to 20 ml. with scintillating solution[8] in crylite vials that were low in potassium. The efficiency of the system was such that the standards delivered 300,000 cts./min./μc. S^{35} at 6° C. The concentration of radioactivity per mg. of thyroid tissue was determined as follows:

$$\frac{\text{cts./ml. thyroid supernatant} \times \text{total volume supernatant}}{\text{thyroid weight}}$$

The T/S ratio was then calculated after appropriate weight and volume corrections:

$$\frac{\text{cts./gm. thyroid}}{\text{cts./ml. plasma}}$$

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[4] Abbott Laboratories, Oak Ridge, Tenn. (potassium thiocyanate-S^{35} shipped with a specific activity of 0.05 mc. to 0.30 mc./mg.).

[5] Armour Thytropar.

[6] Occasionally some of the animals received up to 300 μc. of thiocyanate-S^{35} in order to localize the thyroid fractions following paper chromatography.

[7] Packard Instrument Co., LaGrange, Illinois.

[8] 4 gm. PPO (2,5-diphenyloxazole), 25 mg. POPOP (1,5-di-[2-[5-phenyloxazolyl]] -benzene) and 50 gm. naphthalene dissolved in 1,000 ml. dioxane (p-dioxane, Eastman Organic Chemicals).

Paper chromatography was carried out on thyroids which had been homogenized in 0.5 ml. distilled water. Approximately 20 μg. of tissue homogenate were applied along a 1 cm. line to Whatman No. 1 chromatography paper. The chromatograms were run in an ascending solvent system of butanol, pyridine and 1.5N NH_3 (2:1:2) for 16–18 hours at room temperature. The radioactive zones along the paper strips were localized by means of autoradiography on No-Screen-X-Ray film, and a scintillation strip counter.[9] The unknown radioactive spots were identified by comparing them with thiocyanate and sulfate-S^{35} standards which were run simultaneously. Staining did not prove to be feasible because of the low concentrations of stable sulfate and thiocyanate.

RESULTS

The concentration of S^{35}-labeled material was significantly increased in the thyroid glands of the TSH-treated animals, with the T/S ratios approximately twice as great as those of the controls receiving only thiocyanate-S^{35} ($P < 0.01$) (Table 1). TSH failed to produce increments in thyroidal radioactivity when the guinea pigs were given thiourea prior to the labeled thiocyanate or when sulfate-S^{35} was administered in place of thiocyanate-S^{35} (Fig. 1). However, it should be noted that thiourea by itself effected distinct elevations in the intrathyroidal radioactivity after labeled thiocyanate (Fig. 1). This observation is consistent with the report of Maloof and Soodak (9), as is the finding of negligible amounts of radioactivity within the protein-precipitable fraction of the thyroid homogenates. In most instances this amounted to no more than 2% of the total thyroidal radioactivity.

Positive identification of the labeled compounds within the thyroid glands after paper chromatography was limited to thiocyanate-S^{35} and sulfate-S^{35}. It is not inconceivable that some minor sulfur-containing components with Rf's similar to sulfate-S^{35} would not be separated in this system. Although this seems unlikely, especially in view of other studies (9), it should be understood that sulfate-S^{35} as used here in reference to its derivation from thiocyanate-S^{35} may include other S^{35}-labeled substances. On several occasions radioactivity was detected in areas which did not correspond to known compounds. The radioactivity within the thyroid glands of the controls appeared to be divided unevenly between thiocyanate and sulfate with the former occuping the larger share (Fig. 2). Following administration of TSH the normal pattern appeared to be reversed so that the heavier concentration of radioactivity was localized in the sulfate zone. Attempts to quantitate these differences by means of the strip counter were unsuccessful because of the low levels of radioactivity. However, measurement of the transmission densities[10] of the various film zones revealed that the sulfate-S^{35}/thiocyanate-S^{35} ratio of approximately 0.6 was reversed by the administration of TSH, resulting in a sulfate-S^{35}/SCN-S^{35} ratio of approximately 1.7 (Fig. 2).

[9] Designed by one of us (M.L.M.) and built to specification by Laboratory Associates Inc., Belmont, Mass. See reference 8 for outline of detector unit.

[10] Weston Densitometer model #273.

TABLE 1. EFFECT OF TSH IN THREE DIFFERENT EXPERIMENTS UPON THE THYROID/SERUM RADIOACTIVITY IN GUINEA PIGS RECEIVING THIOCYANATE-S-35

The initial body weights could not be matched with the corresponding final body weights; thus, only the average initial body weight is recorded. $P < 0.01$ in comparisons between T/S of control and TSH-treated groups.

Groups	I				II				III			
	Body weight, gm.		Thyroid weight, mg.	T/S	Body weight, gm.		Thyroid weight, mg.	T/S	Body weight, gm.		Thyroid weight, mg.	T/S
	Initial	Final			Initial	Final			Initial	Final		
Thiocyanate-S-35		270	44.0	0.41						280	32.2	0.72
		290	41.4	0.49						260	28.0	0.88
		260	33.6	0.78		285	35.8	0.63		260	24.2	0.91
		270	30.2	0.69		250	34.2	0.61		250	31.4	1.40
		280	31.8	0.60		300	36.8	1.00		255	26.4	1.00
Average	280	274	36.2±6.1*	0.59±0.15	278	278	35.6±1.3	0.74±0.22	250	261	28.4±3.4	0.98±0.25
Thiocyanate-S-35 plus Thyrotropin		240	40.0	0.70						250	46.0	2.30
		250	44.2	2.18						220	45.8	2.40
		250	56.8	0.94		295	47.6	2.20		210	33.8	2.10
		280	31.0	0.84		260	43.2	1.30		245	37.4	2.80
		240	41.4	1.15		260	40.2	1.20		232	23.0	1.80
Average	273	252	42.68±9.3	1.16±0.59	282	272	43.7±3.7	1.57±0.55	245	231	37.0±9.55	2.28±0.37

* 1 standard deviation.

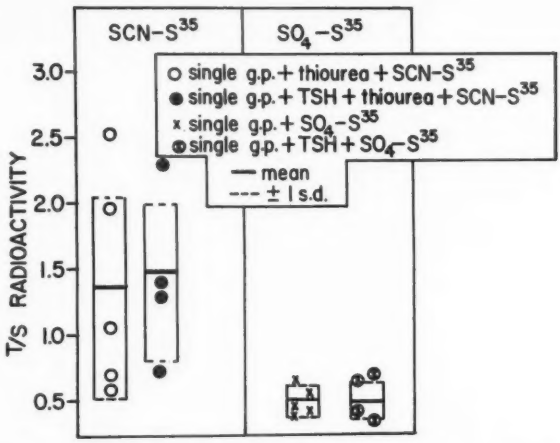

FIG. 1. Failure of TSH to augment the T/S gradient of radioactivity when guinea pigs were pretreated with thiourea or when sulfate-S^{35} was administered in place of SCN-S^{35}. Note the increased T/S radioactivity in both groups receiving the thiourea.

DISCUSSION

In animals injected with thiocyanate-S^{35}, the presence of a gradient of radioactivity between thyroid and plasma in the TSH-treated groups may be explained by the increment of sulfate-S^{35} or other S^{35}-containing compounds. This statement is supported by 1) the increased concentration of S^{35} following TSH and 2) the inability of the TSH to effect a thyroid plasma gradient of radioactivity greater than that of the control group in the presence of thiourea. The latter observation is significant when coupled with the knowledge that sulfate is the major sulfur-containing product in the thyroidal metabolism of thiocyanate and that this metabolism is completely inhibited by thiourea (9).

The elevated T/S concentration gradient of radioactivity for thiocyanate-S^{35} following the administration of thiourea (Fig. 2) resembles to a lesser degree the increment of thyroidal iodide observed in rats pretreated with a single injection of propylthiouracil (10). Since oxidative processes are presumably active in the metabolism of both iodide and thiocyanate ions, thiourea may act upon similar oxidative mechanisms in both instances. It is difficult to explain the differences between our findings and those of Wood and Williams (6). Since their studies were carried out on pooled samples of tissue there is no way of knowing to what extent the variations in gland size or in the concentration of S^{35} radioactivity influenced the mean values. It should also be noted that the animals received considerably greater quantities of stable thiocyanate, as well as being maintained on propylthiouracil for more than one month.

The observations reported here suggest that TSH may enhance the

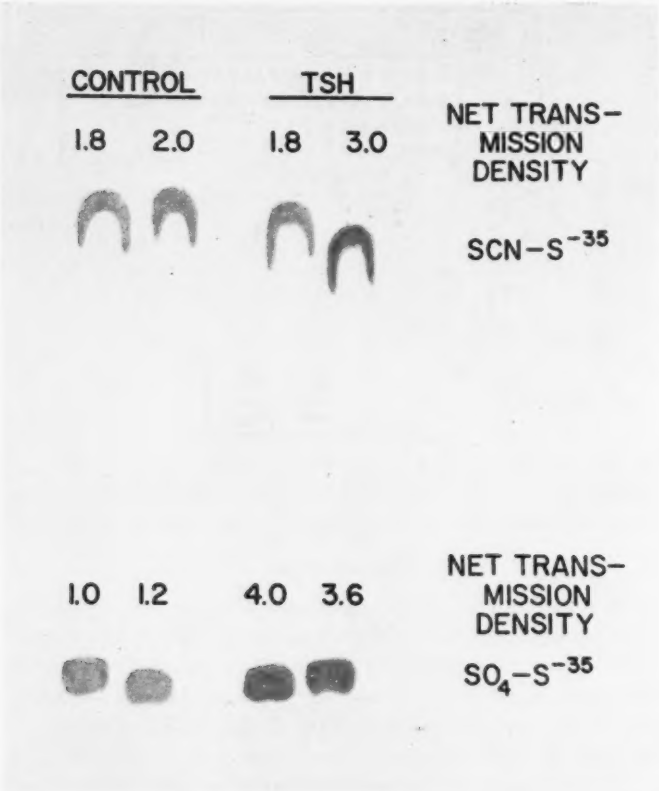

FIG. 2. Retouched radioautograph of chromatogram from control thyroid homogenates on the right and the TSH-treated on the left. Net transmission densities of the thiocyanate-S^{35} and sulfate-S^{35} are recorded above their respective zones. The sulfate-S^{35} represents endogenous breakdown of the exogenously administered thiocyanate-S^{35}.

metabolic degradation of thiocyanate within the thyroid gland. In effect, this would result in a reduced intrathyroidal concentration of thiocyanate with an attendant transfer of plasma thiocyanate into the thyroid cell. The precise locus of action by TSH in the metabolic pathway of thiocyanate is ill-defined. The fact that the T/S gradient of radioactivity seldom varied following the TSH implies the presence of a rate limiting system, perhaps enzymatic in nature. Either acceleration of desulfuration of thiocyanate or the oxidation of the sulfur, as a consequence of the TSH, might account for these results.

The failure of TSH to alter the T/S gradient of radioactivity in the animals receiving sulfate-S^{35} (Fig. 1) indicates that the action of TSH is specific for the intrathyroidal metabolism of the thiocyanate-S^{35}. The pos-

ibility that the absorption or the equilibration of the labeled sulfate was etarded, hence delaying the maximum thyroidal concentration, cannot be xcluded. Allowing longer periods of time for the administered sulfate-S^{35} to circulate might conceivably result in increased T/S gradients of adioactivity.

The results of the experiments described here may elucidate the mechanism whereby TSH induced increments in the thyroidal concentration of radioiodine in the presence of otherwise inhibitory levels of thiocyanate in the normal subjects (7). If the TSH promoted a rapid degradation of thiocyanate, then the intrathyroidal concentration of the thiocyanate ion would have been reduced, consequently relatively normal function would have been restored to the thyroid gland. It is of interest to note that the hyperthyroid glands were less easily inhibited by any given dose of thiocyanate. One might presume a more rapid turnover or transformation of the thiocyanate in thyrotoxicosis not necessarily attributable to TSH.

The presence of low serum concentrations of thiocyanate in some normal subjects (7), presumably from the detoxification of cyanide, attests to a further need to assess the precise relationship of thiocyanate to the function of the normal and abnormal thyroid gland.

Acknowledgment

The authors wish to express their appreciation to Dr. Belton A. Burrows for the use of his laboratory facilities.

REFERENCES

1. Raben, M. S.: *Endocrinology* **45**: 296. 1949.
2. Wolff, J. and I. L. Chaikoff: *Endocrinology* **43**: 174. 1948.
3. Barker, M. H.: *J.A.M.A.* **106**: 762. 1936.
4. Raben, M. S.: *J. Clin. Endocrinol. and Metab.* **13**: 469. 1953.
5. Astwood, E. B.: *J. Pharm. & Exp. Therap.* **78**: 79. 1943.
6. Wood, J. L. and E. F. Williams, Jr.: *J. Biol. Chem.* **177**: 59. 1949.
7. Mitchell, M. L. and M. E. O'Rourke: *J. Clin. Endocrinol. and Metab.* **20**: 47. 1960.
8. Mitchell, M. L. and L. A. Sarkes: *Nucleonics* **14**: 124. 1956.
9. Maloof, F. and M. Soodak: *Endocrinology* **65**: 106. 1959.
10. Vander Laan, J. E. and W. P. Vander Laan: *Endocrinology* **40**: 403. 1947.

EFFECT OF THIOCYANATE, STABLE IODIDE AND PERCHLORATE ON THE KINETICS OF RADIOIODIDE TRANSPORT BETWEEN THYROID GLAND AND BLOOD OF RATS[1]

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ABSTRACT

Equilibration of radioiodide between thyroid and blood was studied in rats fed propylthiouracil with or without subsequent acute administration of thiocyanate, stable iodide or perchlorate. The results were analyzed in terms of Wollman and Reed's open two-compartment model of the thyroidal iodide pump. All three anions examined were found to inhibit thyroidal radioiodide collection predominantly through lowering of the unidirectional clearance of plasma radioiodide by the thyroid, and to a lesser extent by enhancing the exit rate constant of thyroidal radioiodide. These observations are scrutinized for information regarding the anatomical site of the thyroidal iodide pump.

WOLLMAN and Reed (1) investigated the kinetics of radioiodide transport between the propylthiouracil (PTU)-blocked thyroid and the blood of mice and rats. They found that their results could be described by equations based on an open two-compartment model, in which the two variable empirical constants are those of a) the unidirectional clearance of blood radioiodide by the thyroid (C/m_T, in units of μl./mg./min.), and b) the fractional release rate of radioiodide from the thyroid per minute (K_{TB}). The commonly used index of thyroidal iodide pump activity, the equilibrium value of the thyroid-serum radioiodide concentration ratio (T/S), was shown to equal $C/m_T/K_{TB}$. Wollman and Reed reported that the lowering of the T/S by SCN^- is entirely due to an elevation of the K_{TB}. They also suggested, on the basis of experiments performed by others in human beings, that the effect of stable I^- may be of the same nature.

Our study was designed to test the effects of SCN^-, stable I^- and ClO_4^- on the kinetics of thyroidal radioiodide transport in rats chronically treated with PTU. Under the conditions of our experiments depression of the C/m_T was a more striking effect of these ions than elevation of the K_{TB}.

MATERIALS AND METHODS

Young adult male Sprague-Dawley rats were fed 0.03% PTU in Low Iodine Test Diet (Nutritional Biochemicals, Inc., Cleveland, Ohio) for 19–22 days. The rats in ex-

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[1] Aided by U.S.P.H.S. grant A-2463.

[2] N.I.H. predoctoral fellow.

periment I weighed around 200 gm. and those in experiment II about 160 gm. at the time they were killed. All rats were injected subcutaneously (s.c.) with 12 mg. of PTU in alkaline solution 45–60 minutes before the s.c. injection of carrier-free I^{131} (2.5 or 1 μc./100 gm. in experiment I, 2 or 4 μc. in experiment II). The rats in groups b and c of both experiments were also injected s.c., at the time the PTU was given, with the following substances (all dosages/100 gm.; injection volume 0.25 ml./100 gm.): 307 μg. SCN^- (I b), 1,535 μg. SCN^- (I c), 3,293 μg. stable I^- (II b), or 110 μg. ClO_4^- (II c). Groups I a and II a served as controls. The rats were anesthetized with ether, starting $1\frac{1}{2}$–2 minutes before blood was drawn from the abdominal aortas 3, 6, 10 and 30 minutes after the injection of I^{131}. The number of animals per time interval was generally 5 and occasionally 4 or 6. Thyroid samples were quickly removed after exsanguination and weighed on a torsion balance. Both thyroid and serum samples were prepared for counting with a thin mica end-window Geiger-Müller tube, following the method of VanderLaan and Greer (2). When high activity of the thyroid necessitated the injection of lower doses of I^{131} (2.5 or 2 μc./100 gm.), all counts were doubled to make them comparable with those of rats in the same group which received the higher dose, and are thus recorded in Figure 1.

Smooth time curves of serum I^{131} levels (S) were graphically fitted to the values observed, as shown in Figure 1. The smooth time curves for the thyroid I^{131} concentrations (T) displayed in Figure 1 were obtained with an analog computer by simulating the serum I^{131} curves graphed and substituting different C/m_T and K_{TB} values in Wollman and Reed's (1) eq. 1 until the thyroid I^{131} curves that give the best fit were attained.[3] The values of C/m_T and K_{TB} which yielded this optimal fit are shown for each experimental group in Figure 1. The T/S curves presented in Figure 1 connect points determined by dividing values along the T curve by temporally corresponding values along the S curve. The actually measured mean S, T and T/S readings, and their standard errors, are represented in Figure 1 by symbols identified on the graph of experiment I a, and by vertical brackets.

RESULTS

Figure 1 shows that the lower dose of SCN^- (0.5 mg. KSCN/100 gm.) caused a reduction of the C/m_T and an elevation of the K_{TB} (compare I a and b). The higher dose of SCN^- (2.5 mg. KSCN/100 gm.) had little further effect on the K_{TB}, but lowered the C/m_T substantially (I c). When a dose of NaI^{127} equimolar to this higher dose of KSCN (II b) or 1/20 of the equimolar dose of $NaClO_4$ (II c) were given, a comparison with the control group (II a) showed a diminution of the C/m_T to approximately 1/10 the control value, and a rise of the K_{TB} that was less than twofold.

DISCUSSION

Our results do not agree with those of Wollman and Reed (1), who found that 438 μg. of SCN^-/100 gm. did not affect the C/m_T and lowered the T/S by tripling the K_{TB}. In our experimental groups the C/m_T was lowered —very substantially so in all but the group given the lower dose of SCN^-— and the rise in the K_{TB} was not striking. It may be that Wollman and Reed did not observe a decreased C/m_T after SCN^- administration because their

[3] We are greatly indebted by Dr. James O. Osburn (Department of Chemical Engineering) and Dr. Harold W. Shipton and his staff (Medical Electronics) for their invaluable assistance.

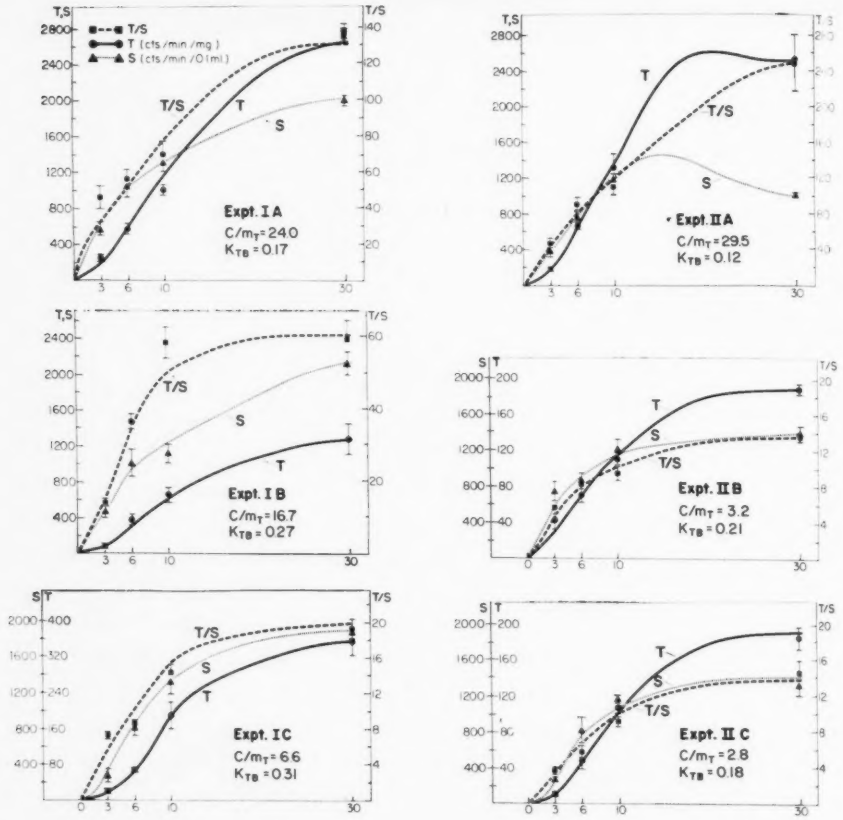

FIG. 1. Effect of various anions on the equilibration of radioiodide between blood and thyroid. The numbers along the abscissae indicate minutes after I^{131} injection. See text for symbols not explained in the figure. I a and II a: control groups; I b: lower dose of SCN^-; I c: higher dose of SCN^-; II b: stable I^-; II c: ClO_4. Dosages are given in Materials and Methods section.

animals, unlike ours, had not received chronic PTU treatment, and their controls therefore did not show C/m_T values as high as those of the control rats in this study. It is very likely that higher doses of SCN^- (or equally effective doses of stable I^- and ClO_4^-) would have depressed the C/m_T under their experimental conditions as they did under ours.

In addition to their two-compartment model of the thyroid, which they used in estimating constants for a variety of experimental conditions, Wollman and Reed also considered (and appeared to favor) a three-compartment model, in which one compartment represents the cells and an-

other the follicular lumen, and C/m_T is a measure of iodide flux from blood into the adjacent end of the thyroid cell. They showed that the three-compartment model yields the same equation as the two-compartment model if iodide concentration is substantially higher in the lumen than in the cell. Whereas, however, in the case of the two-compartment model an increase in the K_{TB}, such as that observed after SCN^- administration, would indicate an enhanced rate of exit of iodide from its thyroidal site of maximum concentration, in the case of the three-compartment model inhibition of iodide transport from cell to lumen would also raise the K_{TB}. Wollman and Reed considered it likely that SCN^- competes with iodide for a carrier at the apical end of the thyroid cell, and that the basal portion of the cell membrane is neither directly involved in the concentration of iodide nor a site of action of SCN^-.

The anatomical interpretation which Wollman and Reed gave their three-compartment model cannot be proved or disproved at this time, but can be used as a working hypothesis. If this is done, however, objections arise to the idea that the base of the thyroid cell is not responsible for the concentration of iodide.

Our finding of a marked reduction of the C/m_T under the influence of SCN^-, stable I^- and ClO_4^- strongly suggests that unidirectional thyroidal iodide clearance does involve transport by carrier, and is only compatible with Wollman and Reed's anatomical interpretation of the three-compartment model if one assumes that the passage of iodide from blood into the base of the thyroid cell is an active process. The presence of another iodide carrier at the apex of the cell is possible, and diminution of its efficiency could be reflected by an augmentation of the K_{TB}. But when the K_{TB} rises after the administration of SCN^-, there is no clear-cut dose-response relationship, nor is this rise nearly as spectacular as the fall of the C/m_T when high enough doses of SCN^-, stable I^- or ClO_4^- are given to depress the T/S markedly. This is hard to reconcile with the idea of an apical iodide carrier that is competitively inhibited by SCN^- and ClO_4^-, and saturated by stable I^-, the same way the basal carrier seems to be.

An alternate hypothesis utilizes Wollman and Reed's two-compartment model and envisages only one iodide carrier site in the thyroid. Doniach and Logothetopoulos's (3) observations suggest that the main region of iodide concentration in the blocked thyroid is the follicular lumen rather than the cell. The single carrier would thus have to be situated in the apex of the thyroid cell. Its activation by TSH, inhibition by SCN^- and ClO_4^-, and saturation by stable I^-, would be largely responsible for the changes in the C/m_T which these substances bring about. Increases in the K_{TB} elicited by TSH, thiouracil, SCN^- (1) or related anions could be due to an enhanced permeability of some part of the thyroid cell or of the surrounding ground substance to iodide leaving the site of its highest concentration

(presumably by diffusion). This hypothesis does not appear to be contradicted by any known facts, and may have at least heuristic merit.

REFERENCES

1. Wollman, S. H. and F. E. Reed: *Am. J. Physiol.* **196:** 113. 1959.
2. VanderLaan, W. P. and M. A. Greer: *Endocrinology* **47:** 36. 1950.
3. Doniach, I. and J. H. Logothetopoulos: *J. Endocrinol.* **13:** 70. 1955.

RELATIONSHIP OF THE ADRENAL MEDULLA TO THE HYPERGLYCEMIC EFFECT OF GLUCAGON[1]

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ABSTRACT

The effect of intravenously administered glucagon (0.4 mg./kg.) on the blood glucose level was studied in normal, adreno-demedullated and dihydroergotamine (DHE)-treated rats. Glucagon produced little or no change in blood sugar values among adreno-demedullated rats, in contrast to the distinct rise produced in normal animals. DHE treatment greatly diminished the hyperglycemic response of normal animals to glucagon injection. The failure of glucagon to induce hyperglycemia in adreno-demedullated or DHE-treated rats was shown not to be due either to lack of initial glycogen stores or to failure of glucagon to induce hepatic glycogenolysis. In a replacement experiment, subcutaneously injected epinephrine (.05 mg./kg.) restored the hyperglycemic response of adreno-demedullated animals to glucagon. It is concluded that depression of the rate of peripheral glucose uptake by epinephrine action is a prerequisite for the hyperglycemic effect of glucagon, though not for its glycogenolytic action.

Glucagon induced a small increase in muscle glycogen among adreno-demedullated animals but not in normal or DHE-treated animals.

THE hyperglycemic response to intravenously injected glucagon has been well documented in humans and in animals of several species, and is used routinely as evidence of the hepatic glycogenolysis induced by this agent. However, the influence of endocrine alterations on this effect of glucagon is not as well established. Burger (1) reported that the bilateral removal of the adrenal glands did not inhibit the hyperglycemic response to glucagon-containing insulin in dogs. On the other hand, Weisburg *et al.* (2) concluded that, in addition to adequate liver glycogen stores, some factor from the adrenal gland was necessary for the hyperglycemic action of glucagon to be evident in rabbits. We have observed that subcutaneous administration of glucagon failed to increase the blood glucose of adreno-demedullated rats, although glycogen depletion occurred (3).

The purpose of the present study was to determine the relation of adrenal medullary activity to the hyperglycemic effect of glucagon.

METHODS

Male Sprague-Dawley rats weighing 200–260 gm. were used. Bilateral adreno-demedullation was carried out via the lumbar approach and the animals were used 8

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days postoperatively. This interval, which is shorter than that used by most investigators, was selected in order to make certain that the experiments would not be vitiated by regeneration of adrenal medullary tissue. The integrity of the adrenal cortex was demonstrated by the fact that the animals did well without saline supplementation and by histological examination of the adrenals in one group of animals.

Rats were fasted for 24 hours, then fed 7.5 gm. glucose per kg. body weight by stomach tube, and 5 hours were allowed for glycogen deposition. Crystalline glucagon,[2] 0.4 mg./2.0 ml./kg. body weight, was then administered intravenously via the tail vein, without anesthesia. Blood glucose values were determined on tail blood before glucagon injection and at 10 to 15-minute intervals thereafter, using the Nelson method (4). Controls received isotonic saline. Liver specimens were obtained under Nembutal anesthesia one hour after glucagon administration and glycogen was determined by the method of Good, Kramer and Somogyi (5), using the Nelson method to determine glucose after hydrolysis. In a preliminary experiment, a comparison of sham-operated to normal animals revealed no differences attributable to surgery; hence for convenience, normal animals were used as controls in the majority of the experiments reported. Controls included normal and adrenodemedullated animals injected with glucagon, as well as animals injected with saline, in all appropriate experiments.

In studies of adrenergic blockade among normal animals, dihydroergotamine (DHE) methanesulfonate (Sandoz), 2 mg./kg. per injection, was administered at 15-minute intervals. The initial intravenous DHE injection was given 5 minutes prior to intravenous injection of glucagon (0.4 mg./kg.) or saline. Subsequent injections of DHE were given intraperitoneally. Blood glucose and liver glycogen values were determined by the methods described above.

The epinephrine used in replacement experiments was Adrenaline Chloride 1:1000 (Parke, Davis & Co.) which, according to the manufacturer, contains less than one part per million of norepinephrine.

RESULTS

The changes in blood glucose values and their standard errors following the intravenous administration of crystalline glucagon to normal and adreno-demedullated rats are shown in Figure 1. The mean initial blood glucose concentrations, in mgm, for each group were as follows: Normal-glucagon, 98.0; adreno-demedullated-glucagon, 89.7; normal-saline, 87.0; and adreno-demedullated-saline, 108.0. In normal animals, glucagon induced a prompt rise in blood glucose, with peak levels reached 20 to 45 minutes after injection. These findings are in agreement with those reported by many other investigators. In adreno-demedullated animals, however, little or no rise in blood glucose occurred. Even the peak value, at 20 minutes, was not significantly different from that of saline-injected normal animals ($0.1 < P < 0.2$). The changes in blood glucose among glucagon-injected demedullated animals are significantly different from those of glucagon-injected normal animals at each interval from 10–60 minutes. The differences from 10–50 minutes were significant at the 1% level, while the difference at 60 minutes was significant at the 5% level. Normal ani-

[2] The glucagon used (lot No. CT-871) was generously supplied by Drs. W. R. Kinsley and O. K. Behrens of Eli Lilly Research Laboratories, Indianapolis.

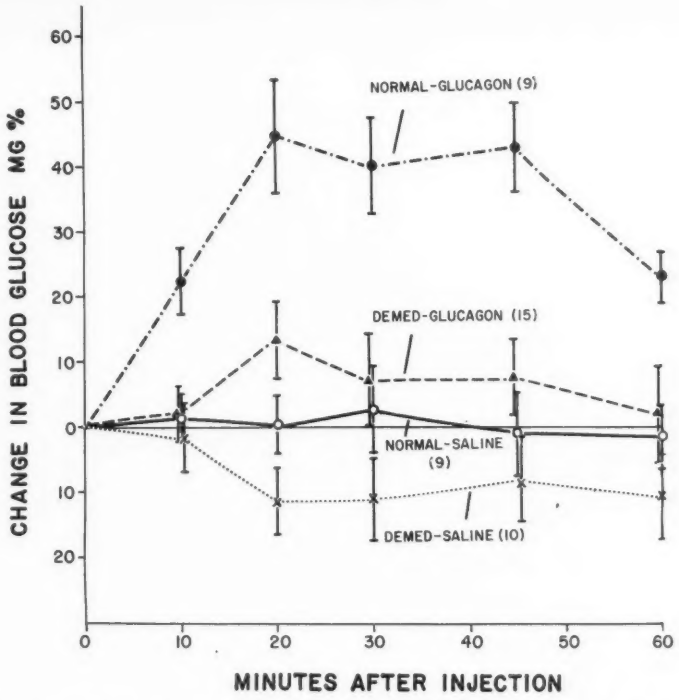

FIG. 1. Comparison of the mean blood glucose changes following intravenous injection of glucagon or saline into adreno-demedullated or normal rats. Figures in parenthesis refer to the number of animals. Vertical lines represent standard errors.

mals injected with saline showed no change in blood glucose, while the adreno-demedullated controls showed a slight decrease.

Table 1 presents the liver and muscle glycogen values of these animals. It is evident that adequate liver glycogen deposition had occurred in both adreno-demedullated and normal control animals. The small difference in liver glycogen levels between normal and adreno-demedullated animals is one we have observed before. In both groups of animals, a marked decline in liver glycogen was found after glucagon injection. Thus, the failure of glucagon to induce hyperglycemia among the adreno-demedullated animals cannot be ascribed either to a lack of liver glycogen initially or to a failure of glucagon in induce hepatic glycogenolysis. It appears, therefore, that presence of the adrenal medulla was necessary for the hyperglycemic effect of glucagon to be manifest.

In the next series of experiments, the effects of adrenergic blockade on glucagon hyperglycemia were investigated. The experiments were conducted as before. Instead of adreno-demedullated animals, normal animals

TABLE 1. LIVER AND MUSCLE GLYCOGEN AFTER GLUCAGON INJECTION INTO NORMAL AND ADRENO-DEMEDULLATED RATS

Experimental group	No. animals	Glucagon dose (mg./kg.)	Time after glucagon admin.	Liver glycogen per cent ±S.E.	Muscle glycogen per cent ±S.E.
Normal—Saline control	9	—	1 hr.	3.19±0.15	0.63±0.04
Normal—Glucagon	9	0.4	1 hr.	0.45±0.13	0.68±0.04
Adrenodemed.—Saline control	10	—	1 hr.	2.56±0.19	0.62±0.05
Adrenodemed.—Glucagon	15	0.4	1 hr.	0.28±0.08	0.67±0.04

treated with dihydroergotamine (DHE), a known adrenergic blocking agent, were used. Controls included animals treated with DHE alone as well as animals injected with saline. The effects on blood glucose are shown in Figure 2. The mean initial blood glucose concentrations, in mg%, for each group were as follows: Normal-glucagon, 80.5; glucagon-DHE, 83.7; normal-DHE, 80.6; normal-saline, 95.8. DHE treatment greatly diminished the hyperglycemic response of normal animals to glucagon injection. The blood glucose levels of the DHE treated animals injected with gluca-

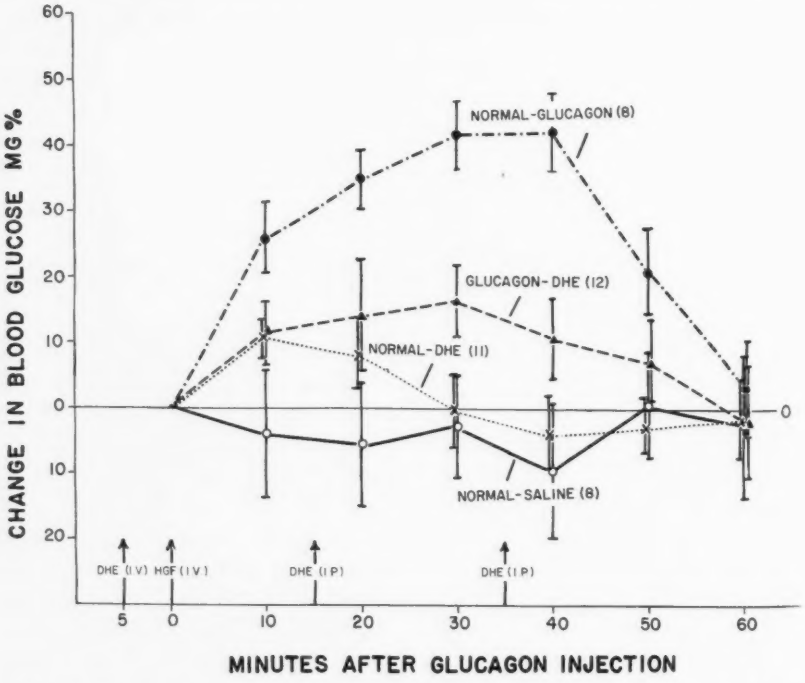

FIG. 2. Comparison of the mean blood glucose changes following intravenous injection of glucagon or saline into normal or dihydroergotamine injected rats. Figures in parenthesis refer to the number of animals. Vertical lines represent standard errors

gon were significantly lower than those of animals receiving glucagon alone at 30 minutes ($P < 0.01$) and at 40 minutes ($0.01 < P < 0.02$). DHE treatment alone had no significant effect on the blood glucose. The results of glycogen analyses are shown in Table 2. Again it is noted that adequate liver glycogen levels were present initially and that glucagon had exerted its usual glycogenolytic effect.

To test the hypothesis that epinephrine secretion is necessary for the hyperglycemic response to glucagon to be manifest, a replacement experiment was carried out, using adreno-demedullated rats. An amount of epinephrine which had been shown by preliminary experiments to produce little or no hyperglycemia or glycogenolysis (0.05 mg./kg.) was injected subcutaneously into adreno-demedullated rats prepared as before. Thirty

TABLE 2. LIVER AND MUSCLE GLYCOGEN AFTER GLUCAGON INJECTION INTO NORMAL AND DIHYDROERGOTAMINE TREATED RATS

Experimental group	No. of animals	Glucagon dose mg./kg.	Dihydroergotamine total dose* mg./kg.	Time after glucagon admin.	Liver glycogen per cent ±S.E.	Muscle glycogen per cent ±S.E.
Normal—Saline control	8	—	—	1 hr.	1.95±0.23	0.55±0.06
Normal—Glucagon	8	0.4	—	1 hr.	0.12±0.07	0.55±0.05
Normal—DHE control	11	—	6.0	1 hr.	2.12±0.22	0.49±0.04
Normal—Glucagon, DHE	12	0.4	6.0	1 hr.	0.14±0.07	0.57±0.05

* Dihydroergotamine methanesulfonate (Sandoz)—Three injections of 2.0 mg./kg./injection.

minutes later glucagon was injected intravenously. Control groups of adreno-demedullated rats received either glucagon alone, epinephrine alone, or saline. A total of four experiments of this type were performed. In three of them, we succeeded in reproducing the blood glucose curve of the normal glucagon-treated animal. The data from these three experiments are presented in Figure 3 and Table 3. The mean initial blood glucose concentrations, in mg.%, for each group were as follows: Normal-glucagon, 110.0; saline-glucagon, 94.4; epinephrine-saline, 111.8; saline-saline, 93.8. Glucagon or epinephrine alone produced only slight transient changes in blood glucose when injected into adreno-demedullated rats. This contrasts with the unequivocal rise in blood glucose produced by a combination of epinephrine and glucagon. Administration of this small dose of epinephrine restored the hyperglycemic response of demedullated animals to glucagon and resulted in blood glucose curves similar to those shown in Figures 1 and 2 for normal glucagon-injected rats. The changes in blood glucose produced by epinephrine plus glucagon were significantly different from the values after injection of glucagon alone at all time intervals from 10–60 minutes ($P < .05$). Adreno-demedullated animals in-

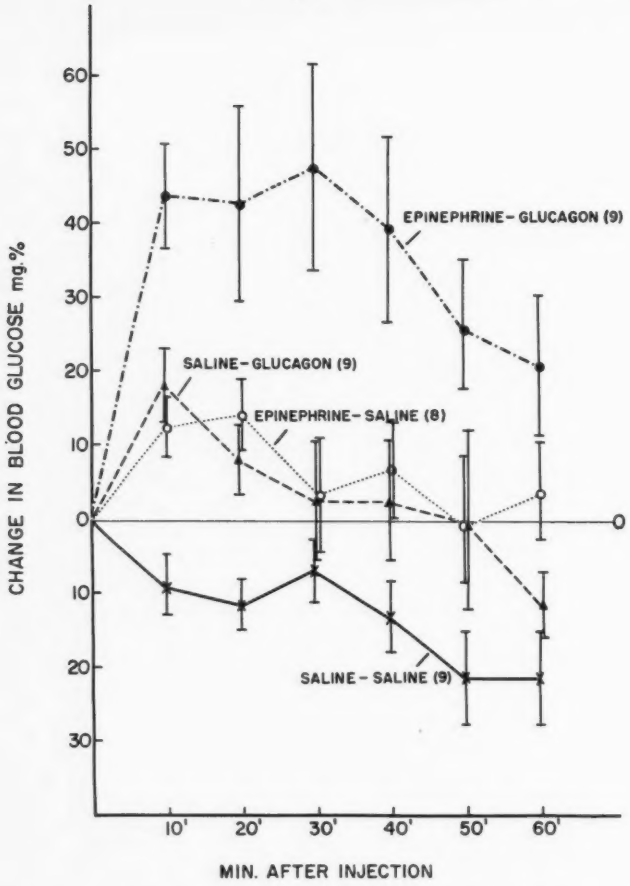

FIG. 3. Comparison of the mean blood glucose changes following injection of glucagon and/or epinephrine into adreno-demedullated rats. Figures in parenthesis refer to the number of animals. Vertical lines represent standard errors.

TABLE 3. LIVER AND MUSCLE GLYCOGEN AFTER GLUCAGON AND/OR EPINEPHRINE INJECTION INTO ADRENO-DEMEDULLATED RATS

Experimental group	No. animals	Epinephrine dose mg./kg.	Glucagon dose mg./kg.	Time after glucagon admin.	Liver glycogen per cent ±S.E.	Muscle glycogen per cent ±S.E.
Saline—Saline	9	—	—	1 hour	2.50±0.15	0.63±0.05
Saline—Glucagon	9	—	0.4	1 hour	0.39±0.13	0.71±0.03
Epinephrine—Saline	8	0.05	—	1 hour	2.47±0.10	0.47±0.07
Epinephrine—Glucagon	9	0.05	0.4	1 hour	0.54±0.12	0.62±0.06

jected with saline showed a continuing decline in blood glucose similar to that seen in Figure 1.

Appropriate muscle glycogen values from Tables 1, 2 and 3 were pooled for statistical analyses. Glucagon did not produce a significant change in muscle glycogen levels of normal animals (Tables 1 and 2), when compared to saline-injected normal controls ($P > .05$). Similarly, no significant change in muscle glycogen was produced by glucagon among DHE-treated animals, as compared to DHE-treated controls (Table 2). Glucagon did, however, induce a small but significant increase ($P < .05$) in muscle glycogen among adreno-demedullated animals, as compared to saline-injected controls. In the replacement experiments with adreno-demedullated animals (Table 3), although epinephrine alone produced no significant change in liver glycogen, it did induce a significant decline in muscle glycogen, as compared to saline-injected controls ($P < .05$). The greater sensitivity of muscle than liver glycogen to epinephrine has been described before (6). It is of interest that when both epinephrine and glucagon were given, the muscle glycogen levels did not differ from those of saline-injected controls.

DISCUSSION

The blood glucose response to injection of glucagon represents a useful and convenient index of the hepatic glycogenolysis induced by this agent. However, it has been demonstrated that rapid hepatic glycogenolysis may occur without a significant elevation of blood glucose (3). Other conditions under which glucagon fails to elicit a hyperglycemic response have been summarized by Weisenfeld (7). The present study has elucidated an additional cause of atypical blood glucose response following intravenous glucagon injection. It is evident from the data in Tables 1, 2 and 3 that failure to observe hyperglycemia following glucagon injection into adreno-demedullated or DHE-treated rats was due neither to lack of initial glycogen stores nor to failure of glucagon to induce hepatic glycogenolysis. Hence, it is concluded that normal adrenal medullary activity is a prerequisite for the hyperglycemic effect of glucagon, though not for its glycogenolytic action.

Based on the diminished hyperglycemic response produced by glucagon in normal rats treated with DHE, a known adrenergic blocking agent, and the observation that exogenous epinephrine can restore the hyperglycemic response to glucagon in adreno-demedullated animals, it appears reasonable to conclude that epinephrine is the hormone required for glucagon hyperglycemia. Our experiments with adreno-demedullated rats satisfy the classic criteria for the demonstration of an endocrine effect. A deviation from the normal was produced by ablation of an endocrine organ, and the normal state was reproduced by administration of the appropriate endocrine product.

Since hepatic glycogenolysis was not inhibited in adreno-demedullated rats injected with glucagon, the action of epinephrine must have been on the periphery, to depress the increased glucose removal that would otherwise accompany glucagon-induced glycogenolysis in these animals. Although there is not complete agreement on this subject, a considerable body of evidence has been presented that epinephrine diminishes the uptake of glucose by the peripheral tissues (8, 9, 10, 11). The arguments for and against this view have been reviewed (12, 13). Also, it has been reported that epinephrine causes a discharge of pituitary and adrenal hormones (14). These hormones are believed to depress the peripheral uptake of glucose (15, 16, 17). Thus, the possibility exists that an indirect mechanism might play some role in decreasing the uptake of glucose in the present experiments. Either a direct or an indirect action of epinephrine would explain our experimental findings.

Epinephrine could play a role in glucagon hyperglycemia in two ways:

(a) It is possible that a normal circulating epinephrine level permits hyperglycemia to occur after glucagon injection by acting as a "brake" against the very rapid increase in glucose disposal which would be required to prevent a sharp rise in blood glucose.

(b) On the other hand, glucagon injection may itself stimulate epinephrine secretion by the adrenal, and the observed effects might be due to increased, rather than normal, circulating levels of this hormone.

The reports that glucagon does not have the effects on blood pressure and pulse that occur after epinephrine injections (18) might appear to militate against a stimulation of endogenous epinephrine release by glucagon. However, Cori *et al.* (19) have examined the relative sensitivity of the blood pressure and the blood glucose level to epinephrine and have demonstrated that hyperglycemia occurs at doses that do not affect blood pressure. Our previous report that subcutaneous injections of glucagon do not induce hyperglycemia in normal animals despite the fact that the magnitude of hepatic glycogenolysis was similar to that produced by intraperitoneal and intravenous injections (3) favors hypothesis (b). If only normal circulating epinephrine levels were needed to obtain glucagon hyperglycemia, such hyperglycemia should be observed after subcutaneous injection as well. It seems likely, therefore, that active epinephrine secretion is stimulated by intravenous or intraperitoneal injections of moderate doses of glucagon, though not by subcutaneous injections. Presumably the latter route of glucagon administration does not result in sufficiently high blood levels to cause adrenal medullary stimulation. Recently, Davidson *et al.* (20, 21) reported that subcutaneous injections of glucagon produced a 35% increase in the oxygen consumption of rats, which was reduced to approximately 12% by adreno-demedullation or by pre-treatment with

DHE. However, the dose of crystalline glucagon injected by these investigators (5 mg./kg.) was approximately 12 times the amount used in our experiments and could easily have resulted in sufficiently high blood levels to cause adrenal medullary stimulation.

The data of Davidson *et al.*, as well as our own experiments, support the suggestion of a relation between glucagon and epinephrine, but the full evaluation of this remains to be determined. However, it is presented as a working hypothesis that intravenous or intraperitoneal injections of glucagon, in the dose range we have used, stimulate the release of epinephrine from the adrenal medulla. With sufficiently high doses, such stimulation will also be induced by subcutaneous glucagon injections. This epinephrine release is not of sufficient magnitude to induce liver or muscle glycogenolysis or cardiovascular effects, but is enough to depress the rate of peripheral glucose utilization so that part of the glucose released by the liver remains in the circulation and a hyperglycemic response is observed.

Ellis *et al.* (22) have presented data indicating that DHE blocks the hyperglycemic effect of epinephrine, but does not interfere with glucagon hyperglycemia, in the rabbit. These data have been widely quoted and DHE is used by many investigators to differentiate between glucagon and epinephrine effects *in vivo*. For example, Sirek *et al.* (23) recently concluded that hyperglycemia produced by blood from the pancreaticoduodenal vein of growth hormone-treated dogs was not due to a glucagon-like substance, since it was abolished by DHE. The results of the present experiments indicate that caution should be exercised in interpreting data when DHE is used to differentiate between the effects of epinephrine and glucagon. Findings similar to those we have observed in the rat might also be obtained in other species, especially if larger doses of DHE are used.

REFERENCES

1. Burger, M.: *Klin. Wchnschr.* **9**: 104. 1930.
2. Weisberg, H. F., Caren, R., Huddestun, B. and R. Levine: *Am. J. Physiol.* **159**: 98. 1949.
3. Sokal, J. E. and E. J. Sarcione: *Proc. Soc. Exper. Biol. & Med.* **98**: 879. 1958.
4. Nelson, N.: *J. Biol. Chem.* **153**: 375. 1944.
5. Good, C. A., Kramer, H. and M. Somogyi: *J. Biol. Chem.* **100**: 485. 1933.
6. Sokal, J. E. and E. J. Sarcione: *Am. J. Physiol.* **196**: 1253. 1959.
7. Weisenfeld, S.: *Proc. Soc. Exper. Biol. & Med.* **97**: 764 1958.
8. Cori, C. F. and G. T. Cori: *J. Biol. Chem.* **79**: 321. 1928.
9. Cori, C. G., Fisher, R. E. and G. T. Cori: *Am. J. Physiol.* **114**: 53. 1935.
10. Somogyi, M.: *J. Biol. Chem.* **186**: 513. 1950.
11. Walaas, O. and E. Walaas: *J. Biol. Chem.* **187**: 769. 1950.
12. Griffith, F. R., Jr.: *Physiol. Rev.* **31**: 151. 1951.
13. Ellis, S.: *Pharmacol. Rev.* **8**: 485. 1956.
14. McDermott, W. V., Fry, E. G., Brobeck, J. R. and C. N. Long: *Proc. Soc. Exper. Biol. & Med.* **73**: 609. 1950.

15. Long, C. N.: *Endocrinology* **30:** 870. 1952.
16. Russell, J.: *Am. J. Physiol.* **140:** 98. 1943.
17. Cohen, J. A.: *Biochim. Biophys. Acta* **4:** 535. 1950.
18. Vuylateke, C. A., DeDuve, C. and A. Nys: *Arch. Int. Physiol.* **57:** 445. 1950.
19. Cori, C. F., Cori, G. T. and K. W. Buchwald.: *Am. J. Physiol.* **93:** 273. 1930.
20. Davidson, I. W., Salter, J. M. and C. H. Best: *Nature* **180:** 1124. 1957.
21. Davidson, I. W. and J. M. Salter: *Fed. Proc.* **17:** 208. 1958.
22. Ellis, S., Anderson, H. L., Jr., and M. Collins: *Proc. Soc. Exper. Biol. & Med.* **84:** 383. 1953.
23. Sirek, O. V., Sirek, A. and C. H. Best: *Am. J. Physiol.* **188:** 17. 1957.

EFFECT OF BRAINSTEM LESIONS ON STRESS-INDUCED CORTICOSTEROID RELEASE IN FEMALE RATS[1]

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ABSTRACT

Five days after unilateral adrenalectomy and placement of brainstem lesions, 16 ♀ rats were subjected to laparotomy and adrenal vein cannulation. Effluent corticosteroid levels, as measured chromatographically and colorimetrically, varied relative to the lesion site. At the rostral pons level, dorsal as well as ventral tegmental lesions were associated with high initial (0–15 minutes) release levels of corticosteroids into the adrenal venous effluent. Dorsal tegmental lesions were associated with an augmented corticosteroid release following acute stress while ventral tegmental lesions inhibited this response. Neither lesion site significantly altered compensatory adrenal hypertrophy.

CHRONIC brainstem lesions at the level of the rostral pons have been found to increase corticosteroids in total 30-minute adrenal venous effluents following acute stress of laparotomy (1). The question arose whether these high levels of corticosteroids recovered represented 1) acute stress-induced increased corticosteroid release, or 2) initially elevated and sustained high circulating levels of steroid due to removal of brainstem areas related to inhibition of ACTH release. In previous experiments, rats given Nembutal (sodium pentobarbital) released more corticosteroids during the 15 to 30-minute adrenal effluent collection period than during the preceding 0 to 15-minute post-cannulation period (2). It was suggested that this phenomenon could be explained partly as an early inhibition of ACTH release by the action of the anesthetic, the increased release in the 15 to 30-minute interval representing a delayed stress-induced response to laparotomy. Sayers (3) has also noted an inhibitory effect of sodium pentobarbital on release of ACTH. The present studies were designed to determine if rats with posterior brainstem lesions given Nembutal exhibited this delayed stress-induced release of corticosteroids in response to laparotomy. In addition an effort was made to assess the possible influence of brainstem areas on hypophyseal mechanisms concerned with the phenomenon of compensatory adrenal hypertrophy.

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MATERIALS AND METHODS

Adult female Sprague-Dawley rats (weighing 200–250 gm.) obtained from our own breeding colony were used in these experiments. Coagulating lesions were placed in the brainstem under conditions as reported previously (1) and immediately the right adrenal was removed and weighed. One control group was included where unilateral adrenalectomy was performed 4 days pre-cannulation; in a second control group the right adrenal was removed at the time of laparotomy. Thus, differences in release levels of corticosteroids from a single adrenal in a state of compensatory hypertrophy could be assessed and compared with release levels recovered from the effluent of a single normal adrenal.

Throughout the entire experimental period rats were placed in individual cages in a temperature-controlled room. All aphagic rats were force-fed 4 times daily with a high protein diet[2] solution supplemented with multivitamins. A 5% glucose-0.9% saline drinking water was offered *ad libitum* and daily fluid volume intake maintained when necessary by force-feeding the drinking solution. Four days postoperatively rats were given sodium pentobarbital (3 mg./100 gm. body weight, i.p.) and 15 minutes later laparotomy and cannulation of the left adrenal vein performed as previously described (2). The total time of surgery was no more than 3 minutes. Effluents were collected in two consecutive 15-minute periods after cannulation. At the termination of the last collection the left adrenal was removed and weighed. Determinations of corticosteroids by chromatographic and colorimetric methods as well as procedures for processing of each brain for reconstruction purposes have been previously described (4). Selections of brain tissue were cut at 80 μ and reconstructions of lesions were mapped on graph paper in sagittal relationship as represented by each 3rd section at a magnification of 10.

RESULTS AND DISCUSSION

1. *Effect of chronic and acute unilateral adrenalectomy on corticosteroid release.* The female rat unilaterally adrenalectomized for 4 days evidenced an adrenal compensatory hypertrophy of 39% (Table 1, Group I). This group released an average 40% additional corticosteroid into a total 30-minute effluent collection as compared to the rat having one adrenal removed at the time of laparotomy (Group II). The major increase (+170) occurred in the 15 to 30-minute collection period. Thus, the hypertrophied adrenal in the chronic unilateral adrenalectomized rat appears capable of maintaining normal secretory levels of steroids as well as synthesizing and/or releasing quantities of corticoids in excess of levels noted for the acutely unilateral adrenalectomized rat.

2. *Effect of chronic unilateral adrenalectomy and brainstem lesions on corticosteroid release.* Data from rats with lesions were divided into 3 categories on the basis of differences in recovery levels of adrenal effluent corticosteroids (Table 1, Groups III, IV and V). A wide range of weight loss and compensatory adrenal hypertrophy was noted in all groups with brainstem lesions but no correlation between these parameters and corticosteroid release seemed apparent (Table 1).

In Group III, levels for each 15-minute collection period were similar

[2] Nutramigen: Protein hydrolysate formula, Mead Johnson.

TABLE 1. EFFECT OF BRAINSTEM LESIONS AND UNILATERAL ADRENALECTOMY ON CORTICOSTEROID RELEASE AND COMPENSATORY ADRENAL HYPERTROPHY IN FEMALE RATS

Group	Total no. rats	Effluent corticoids (total μg.)			% change 15–30 / 0–15 min.	Adrenal wt. % increase left over right	% gm. change body wt.
		Total 0–15 min.	Total 15–30 min.	Total 0–30 min.			
I Chronic ULA*	8	11.9±0.9	33.1±1.4	48.9±2.4‡	+170	39.0±2.0	0
II Acute ULA†	8	13.8±0.8	19.1±1.2	34.9±2.1	+ 39	—	—
III Lesion & ULA*	3	11.8	29.4	41.2	+150	57.3	−29
		10.6	37.0	47.6	+249	48.0	−10
		11.8	29.8	41.6	+164	32.2	−21
IV Lesion & ULA*	3	30.4	41.4	71.8	+ 37	24.6	−15
		27.0	38.6	65.5	+ 40	33.2	−30
		21.0	30.2	51.2	+ 47	23.1	−28
V Lesion & ULA*	10	15.4	13.4	28.8	− 1	34.0	−30
		21.4	8.0	29.4	− 63	24.7	−26
		18.8	7.0	25.8	− 63	32.3	−30
		25.6	21.4	47.0	− 20	44.5	−31
		36.4	21.6	58.0	− 41	30.9	−21
		20.8	4.0	24.8	− 81	43.5	−22
		22.4	22.8	45.2	0	30.0	−30
		19.0	19.6	38.6	0	51.1	−20
		26.6	18.4	45.0	− 31	68.0	−30
		20.2	19.8	40.0	0	37.5	−27

* Unilaterally adrenalectomized 4 days pre-laparotomy.
† Right adrenal removed at laparotomy.
‡ S.E.M. = Standard error of mean.

to those noted in control Group I (chronic unilateral adrenalectomized) (Table 1). The area in which lesions were placed (Fig. 1, A) included that reported previously ineffective in altering total 30-minute release levels of corticosteroids in the female rat acutely unilaterally adrenalectomized (1). Thus, although extensive lesions were placed in the posterior midbrain control levels of corticosteroids were released in response to the stress of laparotomy.

In Group IV and V initial 0 to 15-minute effluent levels of corticosteroids were increased up to 2 to 3 times those noted for control Group I and Group III with lesions (Table 1). Groups IV and V had in common lesions in the region of the dorsal tegmentum at the rostral pons level (Fig. 1, B). This area is comparable in localization with areas in which lesions were reported previously to induce a high or augmented release of corticosteroids in a total 30-minute adrenal effluent collection (1).

If, as previously suggested, the initially lower secretory levels of corticosteroids (0 to 15-minute period) observed in both the chronic and acutely

unilateral adrenalectomized preparations are a result of inhibition of ACTH release by Nembutal acting in part at the anterior pontine level, then one might expect that lesions in this area would also result in depression of corticosteroid recovery levels. But rats in Groups IV and V released *high* levels of steroids for the initial 15-minute period. Therefore, one might hypothesize the following: a) the action of Nembutal is one which selectively inhibits fibers related to a stimulatory effect on release of ACTH, b) in contrast to Nembutal, lesions at the rostral pontine level effectively

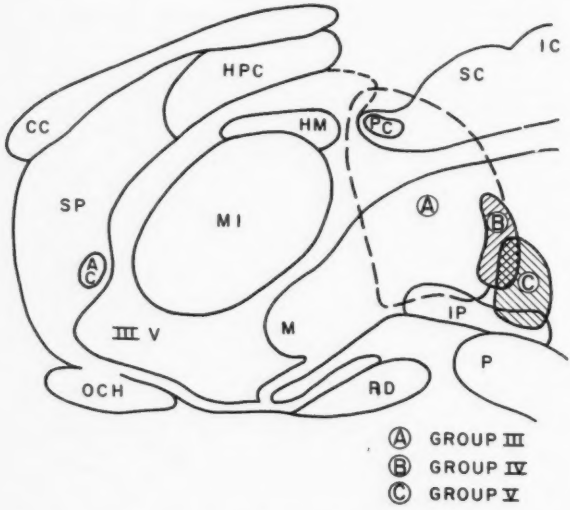

FIG. 1. Localization of brainstem lesions altering stress-induced release of adrenal corticosteroids. Area (A) in dashed outline resulted in normal corticosterone release, (B) resulted in increased release, and (C) resulted in inhibited release. Abbreviations: *MI*, massa intermedia; *HM*, habenulopeduncular nuc.; *SC*, superior colliculus; *IC*, inferior colliculus; *PC*, posterior commissure; *M*, mammillary bodies; *IP*, interpeduncular nucleus; *AD*, adenohypophysis; *CC*, corpus callosum; *HPC*, hippocampal commissure; *SP*, septum, *OCH*, optic chiasma; *AC*, anterior commissure; *P*, pons; *III V;* third ventrical.

remove *both* stimulatory and inhibitory factors. That lesions, however, remove predominately factors concerned with inhibition in the release of ACTH appears evident on the basis of the present data as well as earlier reports. Results from our laboratory (1) as well as the studies by Newman *et al.* (5) have indicated that chronic lesions in rats or acute lesions in cats, placed at the level of the rostral pons, resulted in an augmented release of corticoids into the adrenal venous effluent.

Although corticosteroid levels for the 0 to 15-minute collection period were similar in both Groups IV and V, release levels for the 15 to 30-minute period differed. Whereas Group IV responded to acute stress with an

increased release of corticosteroids (37–47%; Table 1) over initial values, Group V levels of corticosteroids either remained unchanged or were greatly reduced (−81%; Table 1).

The inability of the adrenal in Group V to release increased quantities of steroids for the 15 to 30-minute collection period might be explained on the basis of adrenal exhaustion or an already existing maximal output by the adrenal. That such phenomena are not the answer seems apparent from the fact that in Group V total 30-minute corticosteroid values in most instances were below those noted for rats in other groups with lesions (Table 1). Furthermore, data from Group IV as well as data reported previously for 7 rats having lesions in comparable areas (1) indicate recovery levels of corticoids for the total 30-minute collection period far in excess of the majority of steroid levels noted for Group V.

More specifically, the ability of the adrenal to release additional corticosteroids in response to stress (15 to 30-minute period) appears to be related to the intactness of areas in the central nervous system (CNS) concerned with facilitatory release mechanisms of ACTH. In the present experiments these reinforcement stimuli facilitating ACTH release were surgical stress and hemorrhage (resulting from effluent collections). Thus, the integrity of areas in the brainstem concerned with stress-induced release of ACTH appears to be intact in Group IV but absent in Group V.

Lesions in Group V in addition to including the area in common with Group IV (dorsal tegmentum) included regions in the postero-ventral tegmentum at the level of the rostral pons (Fig. 1, C). Anderson, *et al.* (6) and Sayers (3) have reported that transections of the brain at the pontine level in chronic dog and acute rat preparations respectively, result in an inhibition in the stress-induced release of ACTH in response to a variety of stimuli. Harris (7) has postulated that somatic afferents to the hypothalamus may determine the more discriminatory responses of the hypothalamus while extralemniscal tracts may be responsible for less specific endocrine responses. Thus, the wide range of inhibited responses noted in Group V for the 15 to 30-minute collection period (Table 1) may be directly related to the individual variation of extent of lesion damage to somatic pathways concerned with hypothalamic control of ACTH release.

In view of the fact that numerous afferent and efferent systems connect the midbrain with both the peripheral nervous system as well as more rostral portions of the CNS (8, 9), interruption of specific areas in the midbrain and consequent severance of these pathways would be expected to modify the interdependency of the hypothalamus and lower brainstem in control of ACTH release.

REFERENCES

1. Slusher, Margaret A. and Vaughn Critchlow: *Proc. Soc. Exper. Biol. & Med.* 101: 497. 1959.

2. Slusher, Margaret A. and Sidney Roberts: *Endocrinology* **61:** 98. 1957.
3. Sayers, George: *Colloquia on Endocrinology* **11:** 138. 1957.
4. Beigelman, P. M., Margaret A. Slusher, Grant G. Slater and Sidney Roberts: *Proc. Soc. Exper. Biol. & Med.* **93:** 608. 1956.
5. Newman, Anna E., Edward S. Redgate and Gordon Farrell: *Endocrinology* 63: 723. 1958.
6. Anderson, E., R. W. Bates, E. Hawthorne, W. Haymaker, K. Knowlton, D. Mc.K. Rioch, W. T. Spence and H. Wilson: *Recent Prog. Hormone Res.* XIII: 21. 1957.
7. Harris, Geoffrey: *Reticular Formation of the Brain.* 207. 1958.
8. Nauta, Walle J. H. and Henricus G. J. M. Kuypers: *Reticular Formation of the Brain.* 3. 1958.
9. Feldman, Shaul, Charles S. Van der Heide and Robert E. Porter: *Amer. J. Physiol.* **196:** 1163. 1959.

THYROXINE DEIODINATION BY A MICROSOMAL PREPARATION REQUIRING Fe^{++}, OXYGEN, AND CYSTEINE OR GLUTATHIONE[1]

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ABSTRACT

A microsomal system of liver and kidney is described which deiodinates thyroxine, and which requires Fe^{++} and oxygen. The activity of the system is much enhanced by addition of cysteine, reduced glutathione, or ascorbate.

Deiodination by whole homogenates or by microsomal suspensions is much increased by preheating the tissue to 100° C for a few minutes. Prolonged heating destroys deiodinating activity. Optimal deiodination was observed at an incubation temperature of 37°. The tissue factor has been obtained in an acetone powder and in soluble form.

Dialysis of homogenates against phosphate buffer activates the deiodinating enzyme and makes it thermolabile, but addition of Fe^{++} restores the heat stability of the dialyzed homogenate. Deiodination is inhibited by cyanide, p-hydroxymercuribenzoate, and several substances which chelate iron.

The deoidinating system may belong to a class of reactions which requires Fe^{++}, oxygen, and a reducing agent, and which opens phenolic rings. The tissue factor is thought to be enzymatic in nature because its action is catalytic, it is destroyed by pretreatment with crude trypsin, and because it has tissue specificity.

MONO- and diiodotyrosine are deiodinated by a thermolabile microsomal enzyme of wide distribution which requires TPNH as an essential cofactor (1, 2). During an attempt to extend these findings to a study of the deiodination of thyroxine,[3] it was found that when whole liver homogenate was preheated for purposes of control, the rate at which thyroxine was deiodinated was in fact sharply accelerated as compared to unheated samples. Thermal stability of a thyroxine deiodinating system of liver has been noted previously (3–6).

In this paper we report a deiodinating system in homogenates of liver and kidney which is accelerated by heating at 100° prior to additon of

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[3] The abbreviations, L-T4 and L-T4* are used in the text to refer to stable and I^{131}-labeled L-thyroxine respectively. Tris is Tris (hydroxymethyl) aminomethane.

substrate. An essential participant is in the microsomes, and requires ferrous ion and oxygen. A factor is also required which can be replaced by cysteine, reduced glutathione, or ascorbic acid.

METHODS

Rats of a Sprague-Dawley strain were killed by a blow on the head and the liver quickly minced and homogenized in all-glass tubes at 0°. Sheep liver, thyroids, and kidneys were obtained at the time of slaughter and brought to the laboratory at once on ice. The suspending medium for the homogenates was ice-cold 0.38 M sucrose buffered to pH 7.3 with 0.1 M Tris. The homogenate contained initially 300 mg. whole tissue per ml. Homogenates were spun at 12,000×g for 15 minutes in a refrigerated centrifuge and the supernatant collected. This fraction is referred to below as 12,000×g supernatant. Centrifuging of the 12,000×g supernatant for ½ hour to 1 hour in the Spinco Model L centrifuge at 105,000×g provided the soluble fraction and a microsomal pellet, which was resuspended in cold 0.38 M sucrose buffered with 0.1 M Tris. Phosphate buffer 0.1 M was also used occasionally. In some experiments the microsomes were recentrifuged and suspended several times.

Heated tissues were prepared by immersion of all-glass homogenizing tubes containing the homogenate or fraction into a boiling water bath. The coagulum was made suitable for pipetting by a few turns of the pestle after cooling. Incubation was customarily at 37° for 10 or 20 minutes in a final volume of 4 ml. The reaction was begun by addition of tissue. L-thyroxine labeled with I^{131} in the 3′ position was obtained from Abbott Laboratories and immediately diluted in Tris buffer. Failure to dilute resulted in spontaneous deiodination after several days. The reaction was stopped by addition of 50 μg. of L-T4 to the flask, and immediately thereafter pipetting 0.1 ml. of the reaction mixture into 0.1 ml. of ethanol-ammonia (3:1 v/v) containing unlabeled markers for chromatography. Resolution was on Whatman No. 3 paper. Descending chromatography was used, with a separate slip for each sample. Routinely a solvent system of n-butanol saturated with 2N acetic acid was employed, but identity of the reaction products was confirmed in a solvent system consisting of n-butanol-ethanol (100:20 v/v) saturated with 2N ammonia. The identity of iodide as a reaction product was confirmed by showing that the labeled iodide was extracted by chloroform after addition of iodate at the end of an incubation period.

Zones of labeled iodine located by diazotized sulfanilic acid and palladium chloride were cut from the chromatograms and measured in a well-type scintillation crystal detector. Enough L-T4* was used to give approximately 2,000 counts per minute in a 0.1 ml. aliquot applied to the origin of the chromatogram. Generally only the iodide and T4 zones were cut out and measured, but random strips were counted throughout in order to exclude the possibility of significant reaction products other than iodide. Generally each zone was counted for two or three minutes, the total accumulated counts for the iodide and thyroxine zones being approximately 4,000 to 6,000. Recovery of counts from the chromatogram was in accord with the quantity of labeled iodine initially applied. Occasionally small amounts of labeled iodine were found near the origin and near the solvent front, but this was not a consistent occurrence. The labeled iodine was confined almost entirely to the T4 and the iodide zones. In calculating the fraction of T4 deiodinated, a correction was made for the labeled iodide present in the L-T4* as determined from duplicate or multiple control samples. This varied from 2 to 12%. Controls, always run with each experiment, were either taken at zero time immediately after addition of tissue, or after incubation without addition of tissue. Duplicate or triplicate flasks generally agreed to better than 5%. All flasks, unless otherwise noted,

contained 10^{-3} M thiouracil and Tris buffer 0.1 M at the designated pH. pH values were frequently measured at the end of incubation and found not to vary significantly.

Cysteine HCl and $FeSO_4$ solutions were made fresh for each experiment, either in water or in .05 M HCl. Addition of these in 0.1 ml. volume did not alter the pH of the reaction mixture.

The effects of oxygen and nitrogen were determined in Thunberg tubes. These were prepared by repeatedly evacuating and flushing the tubes with the appropriate gases. The reaction was begun by tipping in the substrate.

The quantity of substrate stable L-T4 added to the incubation flasks varied with the needs of individual experiments, but for the most part was 6.4 mμmoles per flask (5 μg.). The quantity of stable T4 in the added L-T4* was negligible. Without added substrate the deiodination proceeded too rapidly for convenient measurements.

TABLE 1. DEIODINATION OF L-T4 BY FRACTIONS OF LIVER HOMOGENATES

The tissue samples in each flask corresponded to 150 mgm. of rat liver. The soluble fraction was from a 105,000×g centrifugation of a 12,000×g supernatant of whole liver homogenate. The microsomes were resuspended and recentrifuged 3 times. Each flask also contained 10^{-3} M thiouracil, 5 μg. L-T4, and a small amount of I^{131}-labeled L-T4, and 0.1 M Tris buffer to a total volume of 4 ml. pH 7.3. Incubation time, 20 minutes at 37° C. Preheating of tissue samples was for 2 minutes in a boiling water bath. Mean of duplicate flasks.

Tissue preparation	% substrate deiodinated
Microsomes	5.6
Microsomes, preheated	7.0
Soluble fraction	4.3
Soluble fraction, preheated	0.6
Microsomes+soluble fraction	17.8
Microsomes+preheated soluble fraction	48.7
Preheated microsomes+soluble fraction	31.7
Preheated microsomes+preheated soluble fraction	39.2

RESULTS

Heat activation

Heating of whole liver homogenate or the 12,000 g supernatant fraction invariably increased the deiodination of T4 by several fold. The rate of deiodination in unheated 12,000 g supernatant was usually negligible, but occasionally samples were encountered which deiodinated 15 to 20% of the substrate in 20 minutes. The degree of activation was dependent upon the temperature and duration of the preheating. Maximum activation was obtained by heating for 2 minutes in a water bath at 100° C, but partial activation occurred at 60° and 80°. Heating for longer periods of time slowly inactivated the system. There was little change by 20 minutes, but deiodination was much reduced by preheating for 2 hours, and was almost absent if the homogenate was preheated at 100° C for 3 hours.

Heat activation was accomplished by heating either the soluble fraction or the microsomal fraction prior to remixing (Table 1). Neither the heated nor unheated soluble fraction significantly deiodinated the substrate alone.

Minimal deiodination was occasionally observed with unheated microsomes alone.

Deiodination by heated 12,000×g supernatant was significantly reduced by addition of the same amount of unheated supernatant, or by unheated soluble fraction or microsomes. Deiodination was reduced to as much as one-half by these procedures if the heated and unheated samples were preincubated together for 30 minutes prior to additon to flasks containing substrate.

The active fraction of the heated microsomes resided in the heated coagulum. No deiodination occurred when the supernatant from a centrifuged heated sample of microsomes was added to heated or unheated soluble fraction, but the resuspended microsomal coagulum was active if supplemented with heated or unheated soluble fraction. The active component of the heated soluble fraction was present in the supernatant after centrifugation of a heated sample.

Deiodination by heated 12,000 g supernatant was completely inhibited by addition of 0.25 ml. of human serum to the incubation flask. Preheating the serum at 100° C for 2 minutes abolished the inhibiting effect.

Inhibition

Deiodination was completely inhibited by 10^{-3} M $HgCl_2$ and by p-hydroxymercuribenzoate at the same concentration, but not at 10^{-4} M. Several chelating substances inhibited the reaction, among them 5×10^{-4} M Versene, 10^{-3} M penicillamine, 5×10^{-4} M α,α'-dipyridyl, and .001 pyrophosphate, providing it was added after heating or at the instant before heating. Adding pyrophosphate earlier failed to inhibit, presumably because of the action on it of tissue pyrophosphatase. Catalase did not inhibit, but cyanide 10^{-2} M inhibited. A pH optimum between 6 and 7 was observed.

Oxygen dependency

Incubation of preheated homogenate was carried out in Thunberg tubes which were repeatedly evacuated and refilled with nitrogen (Table 2). Air was reintroduced into some of these. Deiodination was always nearly or completely inhibited in the vessels containing nitrogen, and proceeded normally in those to which air had been reintroduced (Table 2). Control vessels of unheated homogenate deiodinated little under air and none under nitrogen.

Temperature dependence

There was a temperature dependence for the deiodination reaction. Little deiodination was observed if the incubation was at 0° C. Deiodination proceeded more rapidly at 37° than at room temperature, and was somewhat less at higher temperature, but still proceeded briskly at 70° C.

TABLE 2. OXYGEN DEPENDENCY OF L-T4 DEIODINATION

The reaction was in Thunberg tubes. Each contained an amount of 12,000×g supernatant of a preheated liver homogenate equivalent to 150 mgm. of rat liver, 2 or 10 μg. of L-T4, a small amount of I-^{131}-labeled L-T4, and 0.1 M Tris to a total volume of 4 ml. The reaction was begun by tipping in the substrates. Incubation for 20 minutes at 37° C. The tubes were repeatedly evacuated and refilled with the appropriate gas. The tissue sample was preheated for 2 minutes at 100°C.

Gas phase	% L-T4* deiodinated	
	2 μg. L-T4	10 μg. L-T4
Air	43.6	28.0
N_2	13.1	2.5
N_2 followed by air	41.3	33.0

Cysteine as an activator

Preheated or unheated microsomes could be activated by a perchloric acid supernatant of the soluble fraction. When it was noted that the PCA supernatant was effective after boiling at pH 1 and was destroyed by storage at pH 10, the possibility was entertained that an active ingredient might be cysteine or a similar substance. This proved to be the case (Table 3). Addition of 10^{-3} M cysteine to heated or unheated microsomes gave excellent activation, but activation was much improved by preheating the microsomes. Similarly, ascorbic acid and reduced GSH in 10^{-3} M concentration activated the microsomes. Several other possible activators of unheated 12,000×g supernatant and heated and unheated microsomes proved ineffective. Among these were DPN, TPN, DPNH, and TPNH. A crude yeast powder[4] was active when added to microsomes.

Fe^{++} as an essential component

Cysteine failed to activate 12,000 g supernatant which had been dialyzed against Versene and then dialyzed free of Versene. When 10^{-4} M Fe^{++} was added in addition to 10^{-3} M cysteine, excellent activation of the Versene-dialyzed homogenate occurred (Table 3). Fe^{++} alone had little effect. No effect was obtained with Fe^{+++}, Co^{++}, Mg^{++}, Mno_4^{++}, Ca^{++}, Mn^{++}, Zn^{++}, Na^{++}, or K^{+}, all in 10^{-4} M concentration.

Dialysis of whole homogenate against phosphate buffer invariably activated the homogenate, and at the same time made it heat-labile (Table 3). The heat lability could be prevented by additon of Fe^{++} prior to heating, but addition of Fe^{++} or cysteine, or both, failed to activate, if done after heating. No explanation for these findings is apparent.

Ferrous ion proved to be effective in activating the unheated 12,000×g homogenate. Activation could be demonstrated in concentrations as low as 10^{-6} M, and optimal activity was reached at 10^{-5} M.

[4] Sigma Chemical Company, St. Louis, Missouri.

TABLE 3. THE EFFECTS OF CYSTEINE, Fe^{++}, AND DIALYSIS

For experimental details see legend for Table 1. Dialysis was overnight. Versene concentration 5×10^{-4} M. These preparations (Exp. C) were dialyzed for 4 additional hours against two changes of 0.1 M phosphate buffer. PCA supernatant: supernatant obtained after perchloric acid precipitation of 12,000×g supernatant of liver homogenate. Mean of duplicate flasks.

Experiment	Tissue	Substances added	% deiodination
A	4× washed microsomes		1.2
	4× washed microsomes	Cysteine 10^{-3} M	38.4
	4× washed microsomes, preheated		3.4
	4× washed microsomes, preheated	Cysteine 10^{-3} M	52.9
B	Unheated microsomes		5.7
	Unheated microsomes	PCA supernatant	44.3
	Unheated microsomes	Ascorbic acid 10^{-3} M	17.0
	Unheated microsomes	GSH 10^{-3} M	10.3
	Unheated microsomes	Cysteine 10^{-3} M	32.4
	Preheated microsomes		8.0
	Preheated microsomes	PCA supernatant	68.5
	Preheated microsomes	Ascorbic acid 10^{-3} M	66.4
	Preheated microsomes	GSH 10^{-3} M	58.6
	Preheated microsomes	Cysteine 10^{-3} M	72.5
	—	PCA supernatant,	> 1.0
	—	Cysteine, GSH,	> 1.0
	—	Ascorbic acid	> 1.0
C	12,000×g supernatant dialyzed against Versene-phosphate buffer		0
	12,000×g supernatant dialyzed against Versene-phosphate buffer	Fe^{++} 10^{-4} M	0
	12,000×g supernatant dialyzed against Versene-phosphate buffer	Cysteine 10^{-3} M	0
	12,000×g supernatant dialyzed against Versene-phosphate buffer	Fe^{++} 10^{-4} M + Cysteine 10^{-3} M	39.3
D	12,000×g supernatant dialyzed against phosphate buffer		24.0
	12,000×g supernatant dialyzed against phosphate buffer	Fe^{++} 10^{-4} M	33.0
	12,000×g supernatant dialyzed against phosphate buffer, heated		0
	12,000×g supernatant dialyzed against phosphate buffer, heated	Fe^{++} 10^{-4} M	2.6
	12,000×g supernatant dialyzed against phosphate buffer	Fe^{++} 10^{-4} M, then heated	26.2

Concentration and solubilization of factors

An acetone powder of the microsomes was obtained which proved to be active in deiodinating L-T4* upon addition of 10^{-3} M cysteine. There was no activity in the supernatant obtained by suspending the powder in Tris buffer and centrifuging. No active preparation was obtained by solubilizing the microsomes in desoxycholate 2%, nor did treatment with butanol (7) solubilize the factor. A neutralized precipitate of microsomes prepared with perchloric acid in 8% final concentration was active when supplemented with 10^{-3} M reduced GSH. When heated microsomes were exposed in the 10 KV sonic oscillator for 10 minutes, and the resulting

supernatant centrifuged for 1 hour at 100,000×g, excellent deiodination was displayed by the supernatant solution when supplemented with GSH. This factor was not dialyzable.

Time course of deiodination

When heated microsomes were incubated with cysteine deiodination did not proceed beyond 10 to 15 minutes, but with GSH the system was much more stable, as can be seen in Figure 1. Deiodination proceeded almost linearly for many minutes.

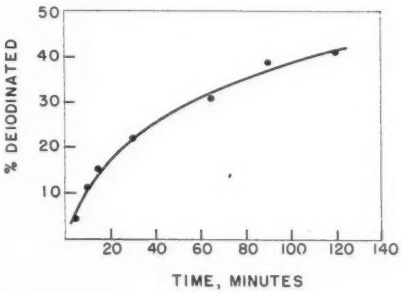

FIG. 1. Time course of deiodination of L-T4 by preheated microsomes supplemented by reduced glutathione 10^{-3} M. 10 μg. L-T4 added to each flask. Points are means of triplicate flasks.

Substrate relationships

The effect of substrate concentration at two levels of concentration of the heated microsomal preparation appears in Figure 2. Each point is the mean of three flasks. Reaction time was 20 minutes. The upper curve is at twice the microsomal concentration of the lower. Points are corrected for the small amounts of iodide found in control flasks at the same substrate concentrations. It is evident that a maximal rate is reached which is proportional to quantity of added microsomal preparation.

Inhibiting effect of crude trypsin

A heated microsomal preparation was adjusted to pH 8.2 and incubated for 4 hours with 10 mg. crude trypsin before readjusting to pH 7.0. Incubation with T4* then gave no deiodination. Control samples treated in the same way but with no added trypsin gave good deiodination, and trypsin added just before incubation with T4* did not inhibit deiodination. Thus a short period of trypsin treatment inactivated the microsomal preparation.

Deiodination by homogenates of other tissues

Most of the observations described above were made on liver. A 12,000× g supernatant of kidney homogenate was activated by preheating, and a preparation of kidney microsomes rapidly deiodinated L-T4* if 10^{-3} M cysteine was added. Minimal deiodination was observed in boiled rat muscle microsomes to which GSH was added. None was observed in thyroid homogenates of microsomal preparations, and none with egg albumin.

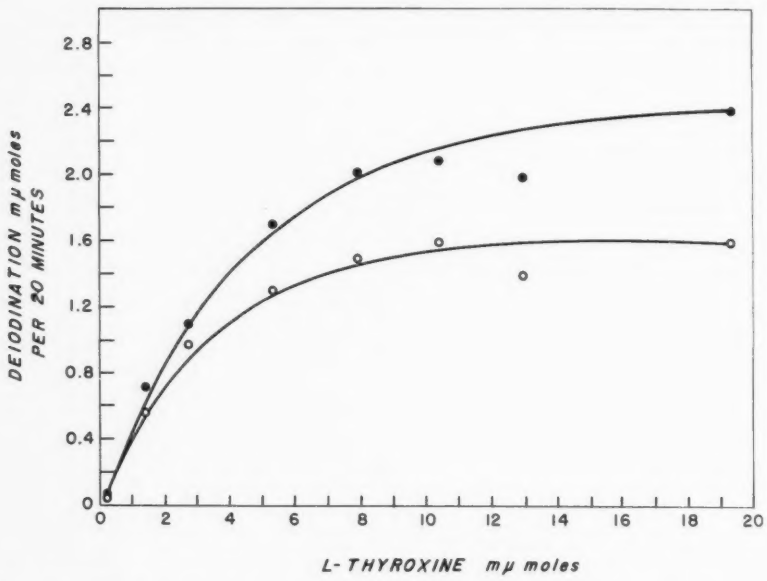

FIG. 2. The effect of varying concentrations of L-T4 on deiodination. The upper curve was obtained from flasks containing preheated microsomes equivalent to 150 mg. of fresh liver, and the lower one containing half as much. Each point is the mean of triplicate flasks. Reduced GSH 10^{-3} M.

DISCUSSION

Deiodination of T4 by heat resistant whole homogenate preparations of rat tissues, including liver, kidney, brain, and muscle, has been described by Maclagan *et al.* (3, 4). A pH optimum of approximately 6.0 was found when L-T4* was employed. Properties of the system included dependence on oxygen, and inhibition by 8×10^{-4} M CN^-, and 5×10^{-2} M citrate. The system was heat-stable for short periods at 100° C, but with prolonged heating, the activity slowly declined and was virtually absent after 2 hours. Others have confirmed the relative heat stability of liver homogenate preparations (5, 6, 8), and deiodination of T4* has been reported after prolonged incubation with boiled kidney slices (9). Lissitzky *et al.* (10) identified thyronine as a product of T4 deiodination by liver slices, but diiodotyrosine was not found in slices incubated with biologically labeled T4 (6, 11). Plaskett (5), on the other hand, has identified an iodinated substance which is easily hydrolyzed to DIT in a homogenate of rat liver incubated with T4. This finding suggests that in the homogenate the β-ring of thyroxine is opened. Tata (12) has reported a preparation from muscle which deiodinates T4, is activated by Fe^{++}, and is further stimulated by flavine nucleotides. Details of the preparation have not yet appeared.

The results which are reported here concern a microsomal preparation

which deiodinates L-T4 under specific conditions. If unheated, it has little activity within the time limit of these experiments. It is thermostable, operates optimally at 37° C if cofactor requirements are met, and is inhibited by Hg^{++}, CN^-, and the chelating substances, citrate, penicillamine, α,α'-dipyridyl, and pyrophosphate. Activity is lost if the microsomes are solubilized with butanol or desoxycholate, but an active acetone powder has been prepared. It withstands perchloric acid and ethanol precipitation. The factor is solubilized to 100,000×g centrifugation by exposure in the 10 KV sonic oscillator.

The action of heat on the homogenate system could be either to produce or activate a specifically required factor, or to destroy an inhibitor. The fact that Fe^{++} activated the unheated homogenate favors the view that the effect of preheating either of microsomes or of supernatant is to release reduced iron in a form which is able to take part in the process of degradation of L-T4*. Inhibition of deiodination by addition of whole serum to an active preparation but not of heated serum supports this view, since unheated serum might be expected to bind or oxidize any free Fe^{++} which might be present. Activation by heat and inhibition by serum could not be explained by T4-binding effects, because the washed microsomes bind T4 very poorly either when heated or not, and the inhibiting amount of serum was far below that which would be saturated by the stable thyroxine in the flask.

Cysteine, or a substance performing a similar role, such as reduced GSH, in general enhanced the deiodinating system. It was required together with Fe^{++} for deiodination in Versene-dialyzed preparations, and it activated washed heated or unheated microsomes without additional Fe^{++}. Oxygen was also required for deiodination. Cysteine potentiated the activating effect of low concentrations of Fe^{++} on an unheated 12,000×g supernatant, but inhibited with high concentrations of Fe^{++}.

Deiodination of iodotyrosines and iodothyronines by oxygen, Fe^{++}, and ascorbate has been extensively studied by Lissitzky and Roques (13), following the demonstration of Udenfriend *et al.* (14) of a similar system for the hydroxylation of many aromatic compounds. The system readily attacks many of the iodinated amino acids, but deiodinates DIT and T4 very slowly. Only approximately 10% of substrate T4 was deiodinated after 12 hours of incubation.

A number of enzymes involved in opening of aromatic rings require oxygen, Fe^{++}, and variously ascorbate, cysteine, or glutathione. Among these are catechol oxidase (15), gentisic acid oxidase (16), and homogentisic acid oxidase (17). It may be suggested that deiodination of T4 in the heated liver microsomal system, requiring as it does oxygen, Fe^{++}, and cysteine (or ascorbate or reduced GSH) may be an analogous ring-opening reaction. This is consistent with the finding of Plaskett referred to above.

The nature of the reaction in the heat-stable liver system is uncertain.

The reaction is clearly not the reversible "deiodination" of thyroxine observed by Tata (18, 19), for that reaction is strongly inhibited by the presence of protein, is reversed slowly with time and instantly by acid, and free iodide is not a reaction product; whereas in the present system ample protein was present, deiodination was progressive with time, an acid solvent chromatographic system was employed, and iodide was proved to be a reaction product.

Several findings are consistent with an enzymatic nature of the reaction. For example, the role of the heated microsomes appears to be catalytic rather than stoichiometric (Fig. 2), since a maximal rate of action was observed. Further, there is a degree of tissue specificity in that excellent deiodination was obtained with liver and kidney, little with muscle, and none with thyroid or egg albumin. Also, the effect of crude trypsin suggests that the catalytic activity is dependent upon specific peptide bonds. The findings provide no information regarding any possible *in vivo* role for the system.

Acknowledgment

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REFERENCES

1. Stanbury, J. B.: *J. Biol. Chem.* **228:** 801. 1957.
2. Stanbury, J. B. and M. L. Morris: *J. Biol. Chem.* **233:** 106. 1958.
3. Sprott, W. E. and N. F. Maclagan: *Biochem. J.* **59:** 288. 1955.
4. Maclagan, N. F. and D. Reid: Ciba Foundation Colloquia on Endocrinology 10: 190, 1957.
5. Plaskett, L. G.: *Nature* **181:** 273. 1958.
6. Yamazaki, E. and D. W. Slingerland: *Endocrinology* **64:** 126. 1959.
7. Hayaishi, O. and A. Kornberg: *J. Biol. Chem.* **206:** 647. 1954.
8. Lissitzky, S., R. Michel, J. Roche and M. Roques: *Bull. Soc. Chim. Biol.* 38: 1413. 1957.
9. Etling, N. and S. B. Barker: *Endocrinology* **65:** 95. 1959.
10. Lissitzky, S., M.-Th. Bénévent, M. Roques, and J. Roche: *C. R. Soc. Biol.* 152: 1490. 1958.
11. Lissitzky, S., M. Roques, M.-Th. Bénévent, and A. Pinchera: *C. R. Soc. Biol.* **152:** 1431. 1958.
12. Tata, J. R.: *Biochim. et Biophys. Acta* **35:** 567. 1959.
13. Lissitzky, S. and M. Roques: *Bull. Soc. Chim. Biol.* **39:** 521. 1957.
14. Udenfriend, S., C. T. Clark, J. Axelrod and B. P. Brodie: *J. Biol. Chem.* 208: 731. 1954.
15. Suda, M., K. Hashimoto, H. Matsouka, and T. Kamahora: *Med. J. Osaka University* **2:** 389. 1951.
16. Douglass, C. D., T. E. Shook, C. L. Weir, and R. Hogan: *Fed. Proc.* **18:** 217. 1959.
17. Knox, W. E. and S. Edwards: *J. Biol. Chem.* **216:** 479. 1955.
18. Tata, J. R.: *Biochem. J.* **72:** 214. 1959.
19. Tata, J. R.: *Biochem. J.* **72:** 222. 1959.

THE ROLE OF THE THYROID GLAND IN THE PRODUCTION OF THE HYPERMETABOLIC STATE OCCURRING IN RATS WITH FULL-THICKNESS BURNS

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ABSTRACT

Thyroid uptake and urinary excretion of I^{131} and water balance studies have been performed upon albino rats with full-thickness burns at four different post-burn time intervals. Rats of the same age and genetic strain were used as controls. The thyroid I^{131} uptakes of the burned rats were not significantly different from uptake values for unburned controls except on the 3rd post-burn day (PBD) when the I^{131} uptake for the burned animals was significantly lower than that for the controls. Except for the immediate post-burn period (3rd PBD) the burned animals excreted less urine and I^{131} during the uptake periods than did the control animals. It is concluded that the burned rat is capable of increased oxygen consumption, *ergo* heat production, with normal or subnormal levels of thyroid activity.

HUMAN patients with full-thickness burns have increased oxygen consumption from the immediate post-burn period until the wounds are closed. However, there is no measurable increment in thyroid radio-iodine (I^{131}) uptake or plasma protein-bound iodine levels in such patients (1).

Approximately two days after having sustained full-thickness burns, albino rats at ambient temperatures of 28° C and below demonstrate an increment in oxygen consumption. This increment reaches a peak with separation or removal of the eschar and then gradually decreases, returning to normal when the wounds are less than 1% of body surface area (2).

This hypermetabolic state occurring in the rat has been demonstrated to have a cause and effect relationship with the increased obligatory heat loss attending the vaporization of the increment in extra-renal water loss through the eschar or open wound (2, 3). Evidence to support this statement is found in the following data:

1. If the trans-wound water loss is prevented from vaporizing by application of a transparent waterproof dressing, the oxygen consumption of the burned animals is no different from that of unburned albino rats (3).
2. If the ambient temperature is elevated to 32° C (one to two degrees above the temperature of the eschar or open wound) the increment in oxygen consumption disappears immediately (2).
3. Thyroidectomized albino rats respond to full-thickness burns with an

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increase in oxygen consumption of the same magnitude as that found in albino rats with intact thyroid glands (2).

These data exclude the possibility that increased thyroid gland activity is essential for such a response in the rat. However, the activity status of the thyroid gland cannot be determined from such data.

Studies of the I^{131} uptake of burned rats have shown an immediate post-burn decrement followed by an increment during the period of positive sodium balance, with a return to normal or subnormal uptake values by the 4th to 5th post-burn day (4). This sequence of events transpires before the prolonged increment in oxygen consumption has barely started. No studies have been made of the thyroid activity during the period of greatest increase in metabolic rate.

The present study reports the activity of the thyroid gland, as measured by I^{131} uptake, for rats with full-thickness burns at four time intervals following burning.

METHODS AND MATERIALS

A total of 64 growing male Sprague-Dawley albino rats was used. These animals were housed in individual cages in an animal room kept at $20° \pm 1°$ C. They were offered Purina laboratory chow and water *ad libitum*. The animals were divided by means of a table of random numbers into four groups containing 16 animals each. These groups were further subdivided randomly into 8 burn and 8 control animals to comprise the experimental population for each of the four post-burn periods investigated.

After the hair from the backs of the animals had been clipped, full-thickness burns of the back measuring $25 \pm 2\%$ of the body surface area were produced by immersion for 30 seconds in water at 90° C. Both experimental and control animals were given ether anesthesia and received intraperitoneal lactate Ringers solution amounting to 10% of body weight.

On four separate occasions—the 3rd, 7th, 21st, and 36th post-burn days (PBD's)—thyroid I^{131} uptake and urinary I^{131} excretion and water balance were obtained for eight control and eight experimental (burned) rats. Selection of animals was made randomly, and on all measurement dates the control and experimental animals for that date were the same age. Staggering the dates on which the burns were produced made it possible for the 3rd and 7th PBD groups and the 21st and 36th PDB groups to have their I^{131} uptake measurements performed on the same dates, thus simplifying the arrangements for and the handling of the isotope. Carrier-free I^{131} was used for the thyroid uptake and urinary excretion studies. By dilution with isotonic sodium chloride the I^{131} was prepared to contain 1 μc. per ml.

The uptake period for all animals was 24 hours. One microcurie of I^{131} was given subcutaneously, immediately following which the animals were placed in individual metabolic cages for the uptake period. The animals were fasted during the uptake period, and urine output and water intake were measured volumetrically.

The animals were sacrificed by a sharp blow to the head and the thyroid glands immediately dissected and placed in one ml. of 30% sodium hydroxide and allowed to digest in a water bath at 90° C. One-tenth ml. aliquots of this solution were allowed to dry on stainless steel planchets. The radioactivity contained therein was estimated by the use of a thin window gas flow Geiger-Mueller tube. One-tenth ml. aliquots of the 24-hour urine specimens were allowed to dry on planchets and counted in the same manner.

One-tenth ml. of the solution used for injection of I^{131} was diluted to one ml. with 30% sodium hydroxide, and to this solution a normal rat thyroid gland was added. After digestion 0.1 ml. of this solution was counted as a standard, and from this the theoretical 100% value for thyroid I^{131} uptake was calculated. Similarly, 0.1 ml. of the solution used for injection of I^{131} was diluted to 1 ml. with normal urine, and by counting 0.1 ml. of this solution the theoretical 100% excretion value for I^{131} was calculated. From these standards the per cent of the given dose of I^{131} taken up by the thyroid and excreted in the urine were calculated for each animal. The results obtained were tested for significant differences by means of the "t" test. The null hypothesis was rejected at the .05 level of probability.

RESULTS

The thyroid uptake and urinary excretion data for I^{131} are summarized in Tables 1 and 2. On the 3rd PBD the I^{131} uptake of the burned animals is significantly lower than that of the controls. On the 7th, 21st, and 36th PBD's there is no significant difference between the I^{131} uptake of the burned and control animals.

The difference in the range of values for thyroid uptake of I^{131} obtained for the 3rd and 7th PBD's compared with values obtained on the 21st and 36th PBD's is not explained by these data. Almost a month elapsed between the obtaining of these two sets of uptake data, and the difference in the age of the animals, variation in iodine content of the food, or changes in unmeasured environmental parameters could all have had some effect. The difference is consistent and uniform for this experiment and does not invalidate the analysis of the I^{131} uptake data.

The I^{131} excretion data and water balance data are presented in Table 3. On the 3rd PBD the burned animals excreted a significantly higher per cent of the given dose of I^{131} and produced a significantly larger volume of urine than the unburned controls. This is the only period during which this obtains, and it may provide the proper explanation for the significantly

TABLE 1. MEANS, STANDARD DEVIATIONS, T RATIOS, AND PROBABILITY VALUES FOR BURNED (E) AND NON-BURNED (C) ANIMALS FOR THYROID I^{131} UPTAKE AT FOUR MEASUREMENT TIMES

	N	Mean	SD	Diff.	t	P-value
3rd PBD						
E	8	2.9	1.5	2.8	2.69	$.01 < P < .02$
C	8	5.7	2.6			
7th PBD						
E	8	8.1	2.9	1.0	.61	NS
C	8	7.1	3.7			
21st PBD						
E	8	2.9	1.1	.6	1.31	NS
C	8	2.3	.6			
36th PBD						
E	8	4.3	2.6	1.1	1.07	NS
C	8	3.2	1.1			

Thyroid I^{131} uptake is expressed as per cent of the given dose.

TABLE 2. MEANS, STANDARD DEVIATIONS, T RATIOS, AND PROBABILITY VALUES FOR BURNED (E) AND NON-BURNED (C) ANIMALS FOR URINARY EXCRETION OF I^{131} AT FOUR MEASUREMENT TIMES

	N	Mean	SD	Diff.	t	P-value
3rd PBD						
E	8	71	11	18	3.40	$<.01$
C	8	53	10			
7th PBD						
E	8	47	13	17	2.68	$<.05$
C	8	64	12			
21st PBD						
E	8	52	8	22	4.01	$<.01$
C	8	74	13			
36th PBD						
E	8	52	13	15	2.23	$.01<P<.05$
C	8	67	14			

I^{131} excretion is expressed as per cent of the given dose.

lower thyroid uptake of I^{131} for the burned animals on the 3rd PBD.

On the 7th, 21st, and 36th PBD's the burned animals excreted a significantly smaller portion of the given dose of I^{131} and produced a significantly lower volume of urine than did the unburned controls. As the eschars mature, the burned animals take in more water and put out less urine than the unburned controls. This seeming paradox is explained by the prodigious quantity of water lost by the burned animals through the eschars or open wounds (2). This has significance in interpretation of the I^{131} thyroid uptake data, since excretion of I^{131} increases as urine volume increases. Thus in spite of retaining more of the given dose of I^{131} during the uptake period, the burned animals do not show a significant increment in I^{131} uptake.

These findings indicate that, if there is any late change in thyroid activity in response to full-thickness burns, it is a decrement and not an increment. These data add to the growing body of evidence that endocrine func-

TABLE 3. MEANS, STANDARD DEVIATIONS, T RATIOS, AND PROBABILITY VALUES FOR BURNED (E) AND NON-BURNED (C) ANIMALS ON WATER INTAKE AND URINE OUTPUT

	Water intake					Urine output					Water balance
	Mean	SD	Diff.	t	P	Mean	SD	Diff.	t	P	
3rd PBD											
E	15.0	8.7	5.0	1.38	NS	28.0	8.3	9.0	2.25	$<.05$	−13.0
C	10.0	5.5				19.0	6.8				− 9.0
7th PBD											
E	9.0	3.0	.4	.18	NS	11.0	3.9	7.0	4.92	$<.001$	− 2.0
C	8.6	6.4				18.0	4.0				− 9.4
21st PBD											
E	20.0	6.6	2.0	.57	NS	10.0	2.9	20.0	6.60	$<.001$	+10.0
C	18.0	7.4				30.0	8.1				− 12.0
36th PBD											
E	21.0	6.2	8.0	2.22	$<.05$	11.0	3.0	9.0	3.85	$<.01$	+10.0
C	13.0	8.1				20.0	5.9				− 7.0

SD = Standard Deviation.
PBD = Post-burn Day.

ion does not control but only modulates the response of the animal to thermal trauma.

Acknowledgment

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REFERENCES

1. Cope, O., G. L. Nard, M. Quijano, R. L. Rovit, J. B. Stanbury and A. Wight: *Ann. Surg.* **137:** 165. 1953.
2. Caldwell, F. T., J. L. Osterholm, N. D. Sower and C. A. Moyer: *Ann. Surg.* **150:** 976. 1959.
3. Lieberman, Z. H. and J. M. Lansche: *Surg. Forum* **7:** 83. 1957.
4. Wase, A. W. and E. Repplinger: *Endocrinology* **53:** 451. 1953.

THYROXINE-SERUM PROTEIN COMPLEXES: SINGLE DIMENSION GEL AND PAPER ELECTROPHORESIS STUDIES

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ABSTRACT

The thyroxine-serum protein complexes have been studied by means of electrophoresis at pH 8.6 using starch gel and paper as a supporting medium. Borate buffer was used in the gel experiments and barbital or ammonium carbonate in the paper experiments. On gel electrophoresis, most humans bind thyroxine in and near the albumin zone. Band 1 corresponds to the fastest moving prealbumin, Band 2 is at the leading edge of the broad albumin zone, band 3 is diffuse and located in the slowest half of the albumin zone and Band 4 travels immediately behind the albumin. Monkeys have a pattern similar to humans but there is a variation in the position of Band 1 and in the fastest moving prealbumin protein which may constitute a polymorphic system. Concomitant variation was found in the mobility of the prealbumin thyroxine complex (TBPA) in paper electrophoresis in ammonium carbonate. In the cow, both Band 1 and TBPA appear to be absent, and other thyroxine-protein bands in starch gel electrophoresis are found which have no counterpart in humans and monkeys.

NORMAL human serum contains several protein components which interact with thyroxine. When equilibrated with human serum at physiological concentrations and subjected to paper electrophoresis in barbital buffer at pH 8.6, I^{131}-labeled L-thyroxine migrates chiefly in a zone between the α_1 and α_2 globulins (1). The protein with which it is considered to interact has been called Thyroxine Binding Protein (TBP) or Thyroxine Binding Globulin (TBG). Smaller amounts of thyroxine migrate with albumin and, much less consistently, in a zone moving faster than albumin ("prealbumin" or TBPA). When tris-maleate (pH 8.6) (2) or other alkaline buffers (*vide infra*) are used, the same three components are found, but the relative distribution of thyroxine is very different; the prealbumin zone in these buffers contains more of the serum thyroxine.

In 1955 Smithies (3) developed a method of electrophoresis using specially prepared starch gel as a suspending medium. The separation of serum proteins in starch gel is apparently dependent on particle size and configuration as well as charge and this results in a separation different from that obtained with other electrophoretic methods. Rich and Bearn (4) applied

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his technique to the study of the thyroxine-protein complexes of human erum. They found that at low thyroxine levels about 60% of the thyroxine as in the leading prealbumin zone and the rest in albumin, suggesting that TBG might migrate faster than albumin in this system. The problem has been re-investigated with the employment of autoradiography for localizing the thyroxine-binding zones. It was found that thyroxine in human serum is located in four separate bands in and near the position of serum albumin. Some human sera have a fifth band. The prealbumin thyroxine-containing band of monkeys was found to travel in three distinct positions in different individuals, and may be an example of a polymorphic trait. A prealbumin thyroxine-binding protein was not present in the cow.

MATERIALS AND METHODS

Blood was collected by venepuncture, allowed to clot at room temperature for four to eight hours, and the sera separated by centrifugation and decantation. The sera was stored frozen at −20° C and thawed slowly at room temperatures just before use. Most of the sera were studied within two to four weeks of collection; however, some of the Indian and American negro sera were studied 8–12 months after collection. Studies to be reported later indicate that thyroxine-binding bands 1–4 are unaffected by storage, while the fifth band (found only in some of the Indians) may be affected by extended storage (see below). The blood samples were collected without regard to time of day, or relation to meals. The human subjects were 17 normal males and 8 normal females from different racial groups, and included 7 American negro males. The ages ranged from 8 to 84 years, but most were young adults. Starch gel and paper electrophoresis runs were done on each of the human sera.

The moneky (*Macaca mulatta*) specimens were collected through the courtesy of Drs. G. L. Van Hoosier and H. E. Rosvold of the National Institutes of Health. The oxen bloods were from the Guernsey herd at the University of Maryland. Blood was obtained from the cows by arterial puncture and from the monkeys by venepuncture. In each case, it was placed in clean dry tubes and, after clotting, the serum removed by centrifugation. Electrophoreses of serum to which thyroxine had been added, followed by autoradiography (see below) was performed with the sera of 13 monkeys and 5 cows. Paper electrophoresis runs with autoradiography (as described below) was also done on each of these. Starch gel electrophoresis runs followed by staining with amido black to reveal the position of the protein bands was performed on a total of 142 monkeys including the 13 monkey sera on which the radiothyroxine studies were done.

I^{131}-labeled L-thyroxine in 50% propylene glycol was obtained from Abbott Laboratories, Oak Ridge, Tennessee. After dilution with water, it was extracted with n-butanol and repurified by an extraction procedure (5). All runs were performed with L-thyroxine which was at least 90% pure as determined by paper chromatography. Mixtures of serum with radioactive L-thyroxine were prepared by adding the serum to the appropriate quantity of vacuum-dried thyroxine (6). The mixtures were allowed to equilibrate for at least 15 hours at 4° C.

Starch gel electrophoresis was carried out by the method of Smithies (3) using the conditions for serum with a pH 8.6 buffer containing 0.03 mole of H_3BO_3 and 0.012 mole of NaOH per liter. After completion of the run, the gel was immediately removed from the tray and index marks were cut from the edges or bored through the thickness of the gel. The filter paper strips which served as the origins were discarded. The gel slab (6 mm. × 80 mm. × approximately 100 mm.) was then split lengthwise, bisecting its

smallest dimension. One half was stained with Amido Black 10B (Hartman-Leddon Co., Philadelphia) dissolved to saturation in a solution containing methanol, water and glacial acetic acid (45:45:10). Excess stain was washed out with solvent alone. The other half of the gel was sealed in "Saran Wrap" and gently pressed between two pieces of "NO-Screen" x-ray film. The entire sandwich was placed in a cold, light-tight box and stored at 4° C in order to reduce diffusion of the protein bands. The length of exposure depended on the radioactivity of the thyroxine and varied from 16 hours to five days. Longer exposures were possible when the gel was kept frozen, but these were less satisfactory. The x-ray film was processed according to the manufacturer's recommendations.

Of the two exposed films, the one placed against the cut surface of the gel, usually gave a superior radioactivity pattern. After contact prints were made, each individual serum pattern was cut out as a narrow strip and the density of the bands was quantitated with an automatic scanning densitometer (Analytrol, Beckman Instrument Co., Palo Alto, Calif.) adapted for such measurement with the B-2 cam. Prior experiments with serial dilutions of a radioiodine solution defined the limit of film darkening below which a linear relationship obtained between the quantity of radioactivity to which the film had been exposed and the area under the densitometry curve. The results were expressed as per cent of total area under the curve. Replicate determinations on the same autoradiograph strip, and on autoradiographs made from the same sera run on different gels, gave good agreement.

The starch gel shrank approximately 15% after staining with Amido Black and washing with solvent. The index marks cut before the two halves of the gels were separated served to correlate the radiothyroxine-containing bands with the stained protein bands. By comparing the index marks, the photographs of Amido Black-stained gel were enlarged to correspond with the prints of the autoradiographs. The gel-halfs which had been used for the autoradiographs did not stain well and were discarded. There was no shrinkage if aqueous bromphenol blue was used for staining, but this gave an inferior definition of the protein bands. Vertical starch gel electrophoreses by the method of Smithies (6) using the borate buffer, were done on six sera drawn from normal white Americans. These six were among the 25 studied by the conventional (horizontal) starch gel method.

Paper electrophoresis was performed with horizontally suspended strips of Whatman 3 MM Filter paper. The reverse flow method (5), was employed for quantitation, both with barbital and ammonium carbonate buffer but the conventional method was also used in some instances. Radioactivity on the paper strips was quantitated with a continuously recording, counting rate meter and a mica-window Geiger-Mueller tube. After radioactivity measurement the proteins were stained with bromphenol blue.

Preliminary experiments with paper electrophoresis were performed with a number of buffers of pH 8.5 to 9 including barbital, phosphate, bicarbonate, borate, trishydroxymethylaminomethane ("Tris"), "Tris"-maleate, glycine, ammediol, ammonium carbonate. In humans a prominent radiothyroxine zone migrating faster than albumin ("prealbumin") was present in all except barbital buffer of ionic strength 0.1. When less concentrated barbital was used ($\Gamma/2 = 0.01$), a consistent prealbumin zone appeared, but this was quantitatively smaller than with the other buffers. The bicarbonate buffer gave variable results due to a rise in pH on the paper strip during the run. This was overcome by the use of ammonium carbonate. Because of its simplicity and the consistently good separations it provided, ammonium carbonate was used in the subsequent experiments.

Ammonium carbonate (U.S.P. grade), 0.1 M, was used. The pH of solutions prepared from different batches of ammonium carbonate varied from 8.35 to 8.8, and the pH was adjusted to 8.3–8.4 by bubbling CO_2 through the solution. After the usual 18 hour

electrophoretic run at a constant current of 2 ma per paper strip (110 to 130 v.) the pH along the paper strip as determined with indicator paper, was approximately 8.5. Fresh solution was prepared each week.

The barbital buffer used in the experiments to be reported below was pH 8.6, ionic strength 0.1

The quantity of thyroxine bound to alpha globulin at two or more concentrations of thyroxine above 0.6 μg./ml. was measured in normal sera separated in the various buffers, and found to be independent of the buffer employed. This concentration of thyroxine is sufficient to saturate TBG when electrophoresis is performed in barbital buffer of 0.1 ionic strength (5). In the case of the other buffers, with the exception of ammonium carbonate, the plateau value (5) for thyroxine bound to TBG was not determined. Thyroxine-binding capacity of TBG may, therefore, be higher in some of these buffers than in barbital, but not lower. In the case of ammonium carbonate, the plateau value was found to be the same as in the barbital system. Plateau values for thyroxine associated with TBPA were not determined.

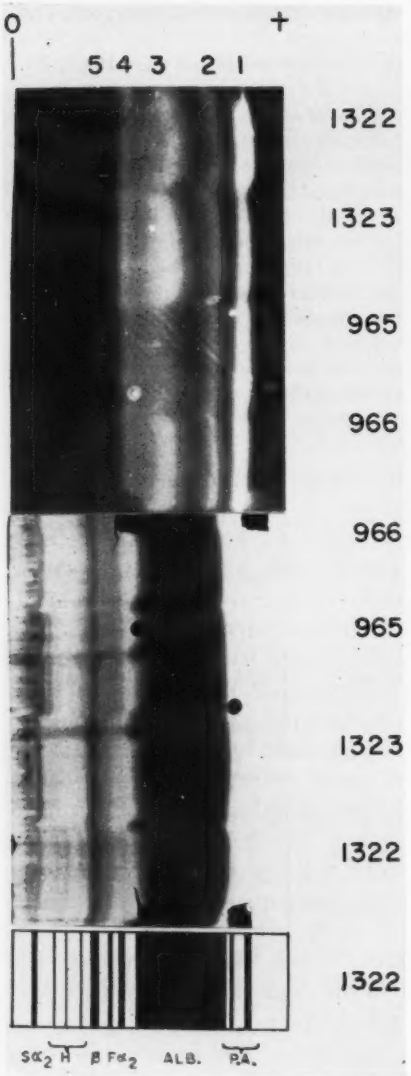
Protein-bound and total I^{127} determinations in the monkey sera were performed by the Boston Medical Laboratory, Boston, Mass., employing the method of Zak (7).

RESULTS

Human. Starch gel electrophoresis of human serum consistently produced four distinct bands of labeled thyroxine in and near the albumin region (Figs. 1 and 2). The fastest-moving band corresponded to the fastest-moving prealbumin (prealbumin 1 or prealbumin A in the nomenclature of Smithies, (3) and Poulik and Smithies (8)). This can be seen from the index marks in Figure 1. The second radiothyroxine band was located at the leading edge of the broad albumin zone. Radioactivity Band 3, which was generally broad and diffuse, was in the slowest half of the albumin zone. The fourth thyroxine band moved just behind the albumin zone and did not appear to coincide with any of the visible protein bands. The position of these bands was confirmed in the vertical starch gel runs. In the absence of serum, some radioactivity moved into the gel several millimeters, but most remained on the filter paper origin. The same four bands were seen when the quantity of thyroxine added to the serum was low (0.075–0.078 μg./ml.) or high (0.68–0.81 μg./ml.), although there were quantitative differences (v.i.).

All the normal human sera had patterns which were qualitatively the same although there was some variation in the relative amounts of thyroxine in each band. The sera of three Alaskan Indians, none of whom were closely related, had an additional slow moving band which travelled in a position near the fast α_2 globulins (nomenclature of Smithies, (3)) and contained less than 10% of the total radiothyroxine. A similar band was seen in other sera which has been stored for several years, and this band may be an artefact.

The results of quantitation of radiothyroxine in the starch gel and on the paper strips are summarized in Table 1A. The quantitative methods are not precise enough to determine the correlation between the paper electropho-



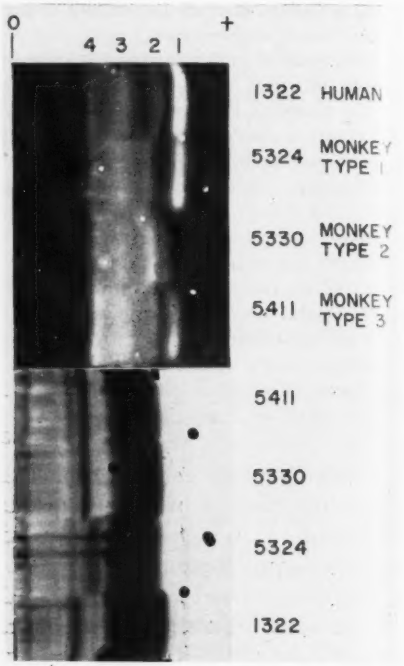


FIG. 2. Distribution of radiothyroxine in monkey sera of Types 1, 2 and 3 compared to human serum. See text and Figure 3. Radiothyroxine in a concentration of approximately 0.74 μg./ml. had been added to these sera.

FIG. 1. Distribution of radiothyroxine in human serum to which 0.68 μg./ml. of radioactive thyroxine had been added. Bottom: starch gel electrophoresis strips stained for protein. The lowest strip is a diagram of the protein pattern. Top: Contact print of the autoradiograph of the same strip. Serum #966 has the fifth slow moving band discussed in the text. O, origin; +, positive pole, P.A., prealbumin; alb, albumin; $F\alpha_2$, fast alpha two globulin; β, beta globulin; H, haptoglobins; $S\alpha_2$, slow alpha two globulin. The diagram is of a haptoglobin type 2-1 serum. The nomenclature is that of Smithies (3).

TABLE 1. DISTRIBUTION OF RADIOTHYROXINE IN FILTER PAPER AND STARCH ELECTROPHORESIS

Thyroxine added	No. determin.		Filter paper				Starch gel		
			TBG		ALB	TBPA	Band 1	Band 2	Bands 3 & 4
			BARB	$(NH_4)_2CO_3$	$(NH_4)_2CO_3$	$(NH_4)_2CO_3$			
(μg./ml.)			%	%	%	%	%	%	%
A. *Human*									
0.075–0.078	3	Range	75–84	42–75	10–27	15–40	6–15	9–10	50–64
		Av.	81	61	18	24	10	10	58
0.68 –0.81	23	Range	13–45	19–50	17–51	19–64	7–35	13–33	29–68
		Av.	33	27	34	38	22	19	53
B. *Monkey, type 1*									
0.073	1		62	45	20	35	19	10	53
0.72 –0.75	3	Range	30–44	24–38	22–34	31–54	20–27	12–17	54–63
		Av.	36	32	28	40	23	15	58

resis and starch gel electrophoresis bands. This has been achieved, in part, by two-dimensional paper-starch gel studies and will be reported elsewhere (11).

None of the bands seen in starch gel electrophoresis contained an amount of radiothyroxine consistently equal to that in any of the paper electrophoresis zones. Bands 3 and 4 were not adequately separated on the densitometry records for accurate quantitation.

"Trailing" of radiothyroxine between Band 4 and the origin amounted to between 14 and 25% of the total at the low thyroxine level, but was less (2 to 11%, with a mean of 6%) at the high thyroxine level.

Monkey. Radiothyroxine patterns in monkey sera were generally similar to the human patterns. There was, however, an intraspecies variation in radiothyroxine Band 1 not seen in humans (Fig. 2). In most monkey sera, the fastest radioactivity band travelled in the same position as in humans, i.e., in prealbumin 1 (Type 1); in some it travelled in a position corresponding to prealbumin 2 (Type 2); and in others in an intermediate position (Type 3). The latter two types appeared to bind less thyroxine in their fastest moving band than did the most common type.

The protein-stained portion of the gel revealed a corresponding variation in the prealbumin bands. This is shown diagrammatically in Figure 3. One hundred and twelve of the 142 animals studied had a major prealbumin band which travelled with the same mobility as human prealbumin 1 and in

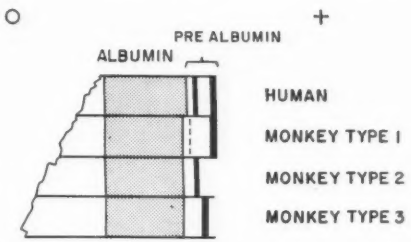


FIG. 3. Schematic diagram of the starch gel electrophoresis strips stained for proteins showing the prealbumin zones of monkey serum compared to human serum. A faint staining slow moving prealbumin is sometimes seen in monkey Type 1 (dotted line). The origin is to the left and the positive pole to the right.

some gel runs a faint band travelling slightly slower than human prealbumin 2 was also seen (Type 1). Rarely (in 5 animals) all of the prealbumin migrated in a position corresponding to the slower human prealbumin (Type 2). In the third variety (25 animals) there was a single faint staining band travelling in a position intermediate between prealbumin 1 and 2. The thyroxine-binding studies were done on representative animals from each of these groups.

A striking variation in the pattern of the serum transferrins (β globulins) on the starch gel was found in the monkeys. At least seven different pat-

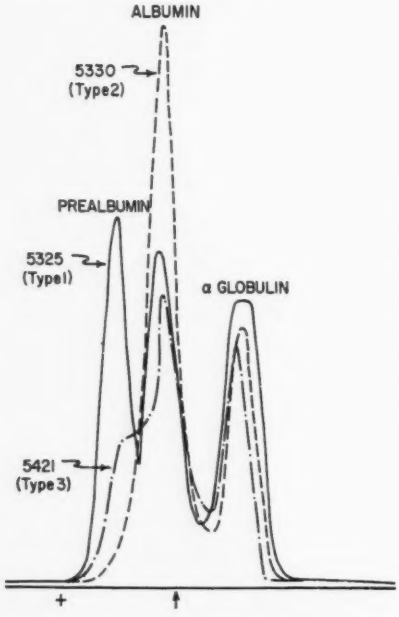


FIG. 4. Distribution of radiothyroxine in monkey sera. Reverse flow paper electrophoresis was performed in 0.1 M ammonium carbonate, and the strips were scanned for radioactivity. Tracings of the radioactivity records are shown. The stained protein patterns were not significantly different from those in humans. The arrow indicates the points of application of the serum and the positive pole is to the left.

terns (which probably constitute a polymorphic system (9)) were demonstrated by means of protein staining and autoradiography with radioiron (10). There does not appear to be any relation between these patterns and the variations in thyroxine binding.

A variation in the fast radiothyroxine zone similar to that observed on starch gel was seen with paper electrophoresis in ammonium carbonate (Fig. 4). The Type 1 pattern was indistinguishable from that seen with human sera. In Type 2, a prealbumin zone was not seen and the radioactivity in albumin was correspondingly increased. In Type 3, the prealbumin radiothyroxine formed a shoulder on the leading edge of the albumin zone.

A variation between the monkey types was also seen with paper electrophoresis in barbital buffer. In Type 1, there was a relatively small shoulder

n the leading edge of the albumin radioactivity peak similar to that fre-quently seen in human serum. This shoulder was absent from Type 2 and Type 3 monkey sera.

Quantitative data are given in Table 1B for monkeys of Type 1. The results were similar to those reported above for humans. Data on monkeys of Types 2 and 3 are omitted, since quantitation of radiothyroxine in TBPA was not possible, but the results were otherwise analogous to those in Type 1.

Serum I^{127} analyses were done in 9 monkeys (3 of each type). The protein bound iodine ranged from 2.8 to 4.2 μg./100 ml. (mean 3.8), and there was no striking difference between the 3 types. The total iodine was from 6.6 to 14.4 μg./100 ml. (mean 9.0), possibly due to high dietary iodine.

Cow. The cow was selected for study as a representative of a group of animals which had previously been found, by paper electrophoresis in barbital buffer, to have a thyroxine-protein pattern resembling that in man (1). This was confirmed in the present experiments. Differences were noted, however, in gel electrophoresis and in paper electrophoresis in ammonium carbonate (Fig. 5). In starch gel, the cow had diffuse bands in the position of human Bands 2 and 3, a band corresponding approximately to human Band 4, and two bands, one easily seen and the other less intense, moving slower than human Band 5. The two slowest bands appear to be in the region of the low β-globulins. Except for a faint trace of radioactivity in one animal, no prealbumin zones were seen either by protein staining or autoradiography. In the ammonium carbonate experiments, the prealbumin radiothyroxine zone was absent and radioactivity was seen only in the albumin and in the "alpha globulin" regions.

DISCUSSION

These findings indicate that the binding of thyroxine to serum proteins may be even more complicated than hertofore believed. Although these studies were performed for the most part at high thyroxine levels, (0.7–0.8 μg. of added thyroxine/ml. of serum) the number of radiothyroxine bands detected when ~0.075 μg. of thyroxine/ml was added was no different. Even at the low thyroxine level, however, the serum contained twice the normal concentration of thyroxine. The use of smaller amounts of thyroxine was not feasible with the available radiothyroxine specific activity. Although the experimental conditions such as thyroxine concentration, extensive dilution of the serum proteins, and unphysiological pH and ionic concentrations make it difficult to apply these findings to the thyroxine-protein combinations which actually exist *in vivo*, they may, at least, help to identify the serum protein species capable of interacting with the thyroid hormone.

There are two indications from the findings reported here that the prealbumin found by starch gel electrophoresis (Band 1) is the same as the prealbumin separated by paper electrophoresis in ammonium carbonate (TBPA). These are 1) a parallel variation in the electrophoretic mobility of

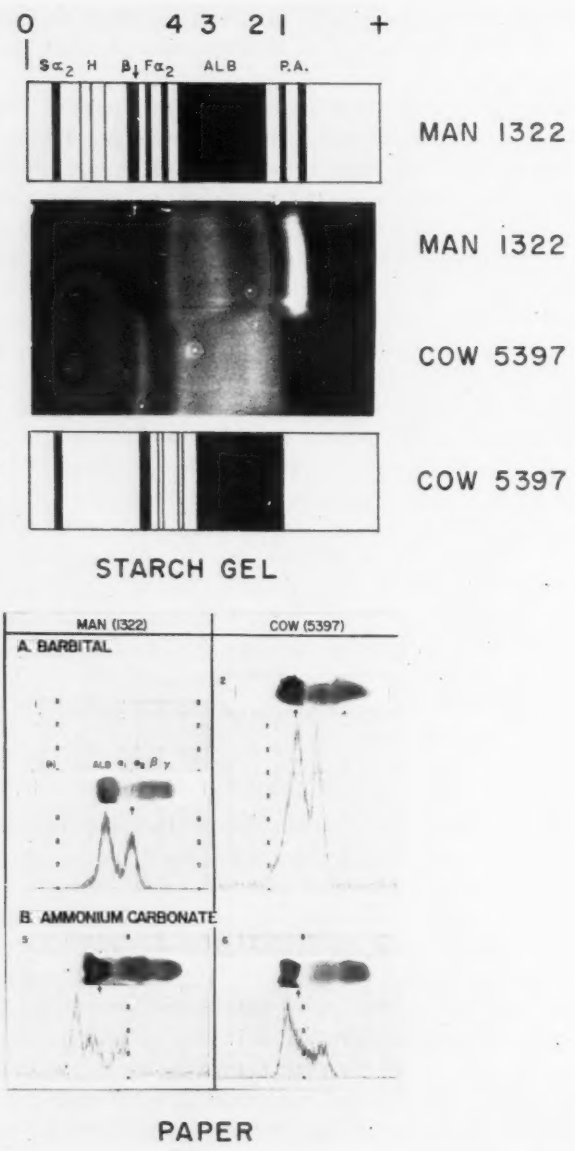


FIG. 5. Comparison of the distribution of radiothyroxine in cow and human serum. *Above* contact print of the autoradiograph after starch gel electrophoresis compared to diagram of electrophoresis strip stained for protein. Two radioactivity bands the slowest very faint, are seen to the left of human radioactivity Band 4. The symbols are the same as in Figure 1. *Below* reverse flow paper electrophoresis of radiothyroxine serum mixtures. The paper strips stained with bromphenol blue are mounted above their

and 1 and TBPA in monkeys, and 2) a parallelism in the presence or absence of Band 1 and TBPA in 3 animal species. In 2 other species not reported here (seal and marmot) both Band 1 and TBPA were absent (11). From the quantitative data presented, however, it is not possible to draw a similar conclusion about the identity of Band 1 and TBPA, and the problem is being investigated further by more direct techniques.

Band 1 corresponds to the fastest of the two prealbumin proteins seen in human serum, confirming the finding of Rich and Bearn (4). It may be noted that the slower prealbumin has been identified as α_1 glycoprotein (8). Earlier experiments (12) indicated that thyroxine did not interact with this protein.

Tata (13) has shown that a prealbumin isolated from serum by Schultz *et al.* (14) is capable of binding thyroxine, and migrates between α_1 and α_2 globulin in the presence of serum in barbital buffer. It seems likely that this protein is not the same as Band 1 or TBPA, as reported here, since the inter-alpha zone in paper electrophoresis contains the same quantity of radiothyroxine at saturation in barbital buffer where TBPA is not seen, and in ammonium carbonate, where TBPA is present. The protein-protein interaction postulated by Tata may, however, be altered by the conditions in the present study. In barbital buffer, TBPA either fails to separate from albumin or loses its affinity for thyroxine. Further studies are required to settle these questions.

It will be of interest to determine whether the thyroxine-prealbumin complex found in cerebro-spinal fluid (1, 15) is the same as TBPA. The former was readily detected with electrophoresis in barbital buffer.

The qualitative and quantitative data reported here suggest a lack of identity between TBG and Band 1. This is in contrast to the reports of Rich and Bearn (4) and Allison (16). By sectioning the gel and counting the radioactivity directly, they found most of the thyroxine in the prealbumin zone even at low thyroxine levels. Rich and Bearn (4), in addition, found that thyroxine isolated with the alpha globulin zone by starch block electrophoresis moved with prealbumin when restudied in starch gel. Allison (16) found in his gel system that the prealbumin of Schultz migrates in the position of the fast prealbumin band. There is no satisfactory explanation the discrepancy between the present work and that reported earlier other for than the likelihood that it is due to technical differences in the performance of the gel electrophoresis.

The identity of Bands 2, 3 and 4 obtained by the techniques employed

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radioactivity record. The arrows indicate the points of application of serum. The amount of radiothyroxine added to the human serum was approximately 0.68 μg./ml. and to the cow serum approximately 0.70 μg./ml.

here has not been established. Band 3 is in a region of the starch gel pattern known to contain α_1 globulin (12), while Band 4 does not coincide with a visible stained protein zone. The non-uniformity of thyroxine in the broad albumin zone is of particular interest. Radiothyroxine Band 2 is in an area not previously identified as a separate protein species. This may be the result of trailing, although in most of the runs it does not have this appearance. It may also indicate an inhomogeneity in the albumin band revealed by thyroxine binding but not by protein staining. This point can only be resolved by isolation and characterization of the bands, and such studies are in progress.

REFERENCES

1. Robbins, J. and J. E. Rall: *Rec. Prog. Hormone Research* **13**: 161. 1957.
2. Ingbar, S. H.: *Endocrinology* **63**: 256. 1958.
3. Smithies, O.: *Biochem. J.* **61**: 629. 1955.
4. Rich, C. and A. G. Bearn: *Endocrinology* **62**: 687. 1958.
5. Robbins, J.: *Archives Biochem. and Biophys.* **63**: 461. 1956.
6. Smithies, O.: *Biochem. J.* **71**: 583. 1959.
7. Zak, B., H. M. Willard, G. B. Myers and A. J. Boyle: *Anal. Chem.* **24**: 1345. 1952.
8. Poulik, M. D. and O. Smithies: *Biochem. J.* **68**: 636. 1958.
9. Ford, E. B.: Genetics for medical students, London, Methuen and Co. 1956. P. 202.
10. Blumberg, B. S.: *Proc. Soc. Exper. Biol. and Med.* **104**: 25. 1960.
11. Blumberg, B. S., L. Farer, J. E. Rall and J. Robbins: *Endocrinology.* In press. 1960.
12. Robbins, J., M. L. Petermann and J. E. Rall: *J. Biol. Chem.* 212 No. 1: 403–410. 1955.
13. Tata, J. R.: *Nature* **183**: 877. 1959.
14. Schultz, H. E., M. S. Schönberger and G. Schwick: *Bioch. Z.* **328**: 267. 1956.
15. Alpers, J. B. and J. E. Rall: *J. of Clin. Endocrinology and Metab.* Vol. XV, No. **12**: 1482–1498. Dec. 1955.
16. Allison, A. C.: *Experientia* **15**: 281. 1959.

STUDIES ON THE POSSIBLE INHIBITORY EFFECT OF CORTICOSTERONE ON CORTICOSTEROIDOGENESIS AT THE ADRENAL LEVEL IN THE RAT[1]

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ABSTRACT

Following the subcutaneous administration of 5 mg. of corticosterone in the rat, the plasma level of this substance was maximal 1 and 2 hours following the injection. In spite of the highly elevated level of circulating corticosterone, no uptake of this substance occurred in the adrenals. The optimal amount of ACTH required to elicit a maximum elevation of adrenal corticosterone two hours after its subcutaneous injection was 2 units. Two hours after the simultaneous administration of 2 units of ACTH and 5 mg. of corticosterone in the intact rat the mean adrenal corticosterone was lower than when 2 units of ACTH were administered alone. When the experiment was repeated with 4 units of ACTH being injected the mean adrenal corticosterone was significantly lower ($P < 0.01$). Repetition of these experiments with ACTH or ACTH plus corticosterone-pretreated hypophysectomized rats indicated that the corticosteroidogenic effect of ACTH was decreased. No ACTH response was observed in rat adrenal sections when these were incubated *in vitro* with 20 μg. of corticosterone and 50 milliunits of ACTH. The experiments suggest that the circulating levels of blood corticosterone in the rat may control its production by acting directly on the adrenal gland by inhibiting the action of ACTH.

IN 1938, Ingle *et al.* (1) reported that one of the major regulatory mechanisms controlling adrenocorticoid production in the rat was mediated via the hypophysis. This humoral theory stated that the amount of circulating corticoid controlled the amount of ACTH[3] produced by the hypophysis. Thus, if a larger than normal amount of corticoid was circulating, the hypophysis would be inhibited and less ACTH produced. Contrarywise, a lower than normal amount of circulating corticoid would release the pituitary from the normal homeostatic control and lead to the well-known fact that more ACTH is produced. This, in turn, triggers the adrenal cor-

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[3] ACTH = Adrenocorticotropin = adrenocorticotropic hormone.

ticosteroidogenic mechanism and leads to an increased corticoid production. Since this original finding, a great number of reports have appeared in the literature which have substantiated the above concept. However, for as long as the above theory has existed, there has been the question as to whether other mechanisms, which can control corticosteroidogenesis, are possible. In support of these possibilities one can cite the apparent existence of an ACTH releasing factor present in the hypothalamus (2, 17, 18). In addition, the possible role of the liver in corticosteroidogenesis has been stressed by Urquhart *et al.* (3), and finally a more direct control of corticosteroid production *in vivo* (4) and *in vitro* (5) at the adrenal level itself has been postulated.

Due to the very small amounts of adrenal steroid present in tissues, and this is true whether an animal it stressed or sedentary (7), it has been very difficult in the past to determine the content of corticosteroids in adrenal tissue. However, the advent of suitable fluorometric techniques permitting the measurement of corticosterone at a very low level of magnitude (0.03 μg.) has opened new avenues which enable a study of adrenal physiology by using individual or a small number of adrenal glands. Thus, it has been possible to analyze the corticosterone content of rat adrenals after administration of steroids and to determine their relative potencies in terms of endogenous ACTH-suppressing activities (8).

In the present investigation, the sensitivity of the fluorometric methods has been exploited to study the action of administered corticosterone in the intact as well as in the hypophysectomized rat stimulated with optimal amounts of ACTH. The results indicate that this steroid inhibits ACTH output and is reflected by a lowered adrenal corticosterone content. By the same criterion it would appear to inhibit the action of administered ACTH *in vivo* at the adrenal level. Corticosterone also inhibited the response to ACTH of rat adrenal glands incubated *in vitro*.

MATERIALS AND METHODS

Animals. Male Sherman rats (150–200 gm.) obtained from Rockland Farms, New York were used for Experiments 1, 2, 3, 4, and 6. In Experiment 5, male Sprague-Dawley rats (100–150 gm.) hypophysectomized by the oral route were used. The latter were obtained from the Charles River Breeding Laboratories, Brookline, Mass.

Measurement of Tissue and Blood Corticosterone. The fluorometric method of Silber *et al.* (6) with the modifications previously proposed (7) was used. All animals were sacrificed by decapitation for reasons given previously (7).

Administration of ACTH and Corticosterone. H.P. ACTHar gel Adrenocorticotropin, a commercial preparation of highly purified ACTH (Armour lot U52804) was used in the *in vivo* experiments. ACTHar lyophylized or ACTHar powder, blend 2-H, a crude ACTH preparation obtained from the Armour Laboratories was used in the *in vitro* experiments. The corticosterone was injected as a suspension in Special Formula (1) at a concentration of 5.0 mg./0.5 ml. in Experiments 1, 2, 3 and 4 and 10 mg./0.5 ml. in Experiment 5. In all cases, the ACTH and corticosterone were injected subcutaneously

TABLE 1. LEVELS OF PLASMA AND ADRENAL CORTICOSTERONE IN THE NORMAL RAT FOLLOWING THE SUBCUTANEOUS ADMINISTRATION OF 5 MG. OF CORTICOSTERONE. EXPERIMENT 1

Time of sacrifice, hours after injection	Number of rats	Average body weight in gm.	Average adrenal weight mg./2 adrenals	Corticosterone	
				μg./100 ml. of plasma, mean ± S.E.	μg./adrenal, mean ± S.E.
Controls	28	154	27.9	22.6 ± 6.6	0.49 ± 0.06
1	8	151	24.0	103.7 ± 17.9	0.19 ± 0.02
2	7	143	22.5	101.5 ± 14.3	0.21 ± 0.02
4	6	152	26.4	65.6 ± 9.0	0.16 ± 0.02
8	8	145	26.1	52.3 ± 6.6	0.17 ± 0.02

in the nape of the neck. When both compounds were injected into the same animal, they were administered on opposite sides of the neck.

EXPERIMENTAL

General

Experiments 1 and 2 were preliminary in nature. They were devised to determine 1) the time relationship between maximum plasma corticosterone levels following the administration of 5 mg. of corticosterone and 2) the amount of ACTH which would cause an optimal or maximal increase in adrenal corticosterone two hours after its injection (7). In Experiments 3, 4, and 5 the effects of administered corticosterone on the response of the adrenal glands to exogenous ACTH was studied in normal and hypophysectomized rats. Experiment 6 was the counterpart of Experiments 3 and 4 wherein the same effects were studied *in vitro*.

Experiment 1. Plasma and adrenal corticosterone levels after a single injection of 5 mg. of corticosterone. In this experiment 57 rats were used of which 28 served as controls and 29 received 5 mg. of corticosterone. The rats were killed in groups (Table 1) 1, 2, 4, and 8 hours after the injection. The plasma corticosterone level was determined in each individual plasma sample while the tissue corticosterone was measured in each pair of adrenals.

Experiment 2. Adrenal corticosterone content in normal rats 2 hours following the injection of ACTH. One hundred and fifty-nine rats were divided into 12 groups (Table 2.)

TABLE 2. ADRENAL CORTICOSTERONE CONTENT IN THE NORMAL RAT TWO HOURS AFTER THE SUBCUTANEOUS INJECTION OF VARIOUS DOSES OF ACTH. EXPERIMENT 2

ACTH dose, U.*	Number of rats	Average body weight in gm.	Average adrenal weight, mg./2 adrenals	μg. of corticosterone/adrenal, mean ± S.E.
Control	23	153	27.7	0.34 ± 0.05
0.4	6	196	30.7	0.39 ± 0.09
0.6	6	202	28.7	0.46 ± 0.10
0.7	6	133	21.9	0.57 ± 0.11
0.8	12	166	27.4	0.78 ± 0.12
1.2	12	166	28.6	0.68 ± 0.11
1.4	12	181	28.2	0.66 ± 0.11
1.6	13	178	28.4	0.83 ± 0.14
2.0	32	172	28.0	0.99 ± 0.07
2.8	5	137	21.8	0.95 ± 0.12
4.0	26	132	24.7	0.91 ± 0.06
8.0	6	126	24.9	1.08 ± 0.04

* International Units.

Eleven groups were injected with 0.4, 0.6, 0.8, 1.2, 1.4, 1.6, 2.0, 2.8, 4.0, and 8.0 units (U.) respectively of ACTH gel. The twelfth group served as control and received an injection of Special Formula equal in volume to the largest ACTH injection. The animals were killed 2 hours after the injections and the corticosterone content measured in each pair of adrenals.

Experiment 3. Adrenal corticosterone level in intact rats 2 hours following the administration of 1) 2 U. ACTH, 2) 2 U. ACTH plus 5 mg. corticosterone and 3) 5 mg. corticosterone. Thirty-two rats were used for 1), 31 for 2), and 11 for 3) (Table 3). Twenty-eight animals which served as controls were injected with volumes of Special Formula equivalent to the largest injection volume used. The animals were killed 2 hours after the injections and the corticosterone content measured in each pair of adrenals.

TABLE 3. THE EFFECT OF ACTH ON THE CONTENT OF ADRENAL CORTICOSTERONE IN THE NORMAL RAT IN THE PRESENCE OR ABSENCE OF ADMINISTERED CORTICOSTERONE

Experiment	Protocol	Number of rats	Average body weight in gm.	Average adrenal weight, mg./2 adrenals	μg. of corticosterone/adrenal, mean ± S.E.	P values
3	ACTH, 2 U.	32	172	28.0	0.99 ± 0.07	0.10
	ACTH, 2 U. + Corticosterone, 5 mg.	31	171	26.7	0.83 ± 0.07	
	Corticosterone, 5 mg.	11	140	27.1	0.20 ± 0.02	<0.05
	Controls	28	164	26.6	0.39 ± 0.05	
4	ACTH, 4 U.	26	131	25.4	0.91 ± 0.06	<0.01
	ACTH, 4 U. + Corticosterone, 5 mg.	26	132	24.7	0.61 ± 0.09	
	Corticosterone, 5 mg.	19	132	26.8	0.19 ± 0.03	<0.01
	Controls	25	131	26.1	0.42 ± 0.05	

Experiment 4. The protocol of this experiment was identical to Experiment 3 except that 4 U. ACTH were used instead of 2 U. Twenty-six animals were used for 1), 26 for 2), and 19 for 3) (Table 3). Twenty-five animals served for the controls.

Experiment 5. Adrenal corticosterone level in hypophysectomized rats following repeated injections of ACTH and ACTH plus corticosterone. Preliminary studies showed that the corticosterone content of newly hypophysectomized rats varied greatly from one animal to another either in groups of rats receiving no ACTH or those groups receiving ACTH. For this reason, it was decided to wait until the basal levels of adrenal corticosterone were constant within a fairly large number of rats. A period of at least 6 days after hypophysectomy was found necessary to fulfill these requirements in rats receiving no ACTH. However, after this period it was found that the rats did not respond in any uniform manner to a level of ACTH (1 U.) which was sufficient to cause unequivocal changes in the adrenal corticosterone content immediately following hypophysectomy. The effects of ACTH injections and ACTH plus corticosterone in several groups of these animals was next investigated. This was done to overcome the known latent period between the time of ACTH administration and the adrenal response with respect to corticoid production in hypophysectomized animals (9). Thus, it was expected that groups receiving daily ACTH injections for several days would have a greater corticosterone level than control groups, those receiving corticosterone plus ACTH, or those

receiving corticosterone alone. The following protocol was carred out. Six days following hypophysectomy, 51 Sprague-Dawley rats were divided into five groups (Table 4). Three groups (a, b, c, *vide infra*) were injected twice daily (8:30 A.M. and 6:30 P.M.) for a period of four days and the animals sacrificed 14 hours following the last injection. The adrenal corticosterone level was determined in each pair of adrenals. The last two groups (d and e) were treated as above except that they received one more injection on the 5th day at 8:30 A.M. and were sacrificed 2 hours after the last injection.

Group (a) Control group—10 animals injected with Special Formula. Sacrificed on the morning of the fifth day.

Group (b) ACTH—corticosterone—14 hours group—10 animals injected with 1 U. of ACTH per injection. Sacrificed 14 hours following the last injection.

TABLE 4. THE EFFECT OF ACTH ON THE CONTENT OF ADRENAL CORTICOSTERONE IN THE HYPOPHYSECTOMIZED RAT IN THE PRESENCE OR ABSENCE OF ADMINISTERED CORTICOSTERONE. EXPERIMENT 5

Groups*	Number of rats	Average body weight in gm.	Average adrenal weight, mg./2 adrenals	μg. of corticosterone/adrenal, mean ± S.E.	P values
(a) Control	7	118	11.7	0.07 ± 0.01	
(b) ACTH, 14 hours	10	123	19.6	0.17 ± 0.02	<0.02
(c) ACTH-Corticosterone, 14 hours	10	109	18.7	0.10 ± 0.03	
(d) ACTH, 2 hours	11	115	19.5	0.38 ± 0.02	<0.01
(e) ACTH-Corticosterone, 2 hours	13	104	19.7	0.30 ± 0.04	

* See Experimental, Experiment 5.

Group (c) ACTH—corticosterone—14 hours group—10 animals injected with 1 U. ACTH plus 10 mg. corticosterone per injection. Sacrificed 14 hours following the last injection.

Group (d) ACTH—2 hours group—11 animals injected with 1 U. ACTH per injection for 4 days and receiving 0.5 U. ACTH on the morning of the 5th day. Sacrificed 2 hours following the last injection.

Group (e) ACTH—corticosterone—2 hours group—13 animals injected with 1 U. plus 10 mg. corticosterone per injection for 4 days. As with group (d) the animals in this group received a final injection on the morning of the 5th day, consisting of 0.5 U. ACTH and 10 mg. corticosterone. Sacrificed 2 hours following the last injection.

Experiment 6. The effect of ACTH in the presence or absence of corticosterone on rat adrenal glands incubated in vitro. Twenty-eight rats were sacrified (Table 5). Following extirpation of the glands these were cleaned of adherent fat. The adrenal glands of each rat were kept paired, then re-paired by cross-over design in order to obtain a uniform distribution of tissue. Each gland was then sectioned into 8 equal sections. The sections from each paired gland were weighed and preincubated in a 20 ml. beaker in a Dubnoff metabolic shaking incubator according to method of Saffran *et al.* (10). A one hour pre-incubation in 2 ml. of Krebs-Ringer bicarbonate-0.01 M glucose medium at 37° C under an atmosphere of 95% O_2-5% CO_2 was carried out. After the pre-incubation

TABLE 5. THE INHIBITION OF ENGODENOUS CORTICOSTERONE PRODUCTION BY CORTICOSTERONE ADDED TO ADRENAL SECTIONS INCUBATED *in vitro* IN THE PRESENCE OF ACTH. EXPERIMENT 6 (see addendum)

Groups*	Average adrenal tissue weight, mg./flask	μg. of corticosterone, mean ± S.E.	P values
Control (group 4)	28.7	3.73 ± 0.28 per 100 mg. tissue	0.001
ACTH (group 1)	26.1	7.19 ± 0.49 per 100 mg. tissue	
Tissue plus Corticosterone (group 3)	26.7	19.75 ± 0.39 per flask	0.30
Corticosterone plus ACTH (group 2)	25.4	20.24 ± 0.34 per flask	

* See Experimental, Experiment 6.

the medium was removed and the beakers divided into four groups of seven beakers. The protocol for the final one-hour incubation was as follows:

	Group 1, ml.	*Group 2*, ml.	*Group 3*, ml.	*Group 4*, ml.
Krebs-Ringer-bicarbonate 0.01 M glucose	1.6	1.6	1.6	1.6
0.05 u. ACTH/0.1 ml. 0.154 M Nacl	0.1	0.1	—	—
20 μg. of corticosterone in 0.1 ml. 100% Ethanol-0.154 M NaCl (1:9 V/V)	—	0.1	0.1	—
100% Ethanol-0.154 M NaCl (1:9 V/V)	0.1	—	—	0.1
0.154 M NaCl	0.2	0.2	0.3	0.3

After the final incubation, the beakers were placed in cracked ice and the contents of each was homogenized in an all glass Potter-Elvejehm homogenizer. The beakers and homogenizer were rinsed with several portions of 17% ethanol which were then added to the homogenate and then made up to a final volume of 10 ml. exactly with 17% ethanol. The final concentration of ethanol was 13.6% which is that concentration of ethanol normally present in homogenates of adrenal tissue prior to the fluorometric analysis of corticosterone (6). The total corticosterone (tissue and incubation medium) in each beaker was then determined by taking suitable aliquots of the ethanolic homogenate (7).

RESULTS AND COMMENTS

Experiment 1. As can be seen from Table 1, the plasma corticosterone levels following the subcutaneous injection of 5 mg. of corticosterone reach maximum values of 103.7 ± 17.9 and 101.5 ± 14.3 μg. per 100 ml. of plasma at the first and second hour respectively following the injection. The average value in the control animals was found to be 22.6 ± 6.6 μg. per 100 ml. of plasma. The values for adrenal corticosterone were found to be 0.49 ± 0.06 μg. per adrenal in the control group while 0.19 ± 0.02, 0.21 ± 0.02, 0.16 ± 0.02 and 0.17 ± 0.02 μg. per adrenal respectively was found in the groups killed 1, 2, 4, and 8 hours respectively following the injection (Table 1). The differences between the control group and the injected groups in terms of adrenal corticosterone content were statistically significant (P values <0.02, 0.05, 0.01, and 0.02 for groups killed 1, 2, 4, and 8 hours respectively). The above results permitted two conclusions. Firstly, that admin-

stered corticosterone did not accumulate in the adrenal and secondly, that ts administration appeared to cause an inhibition of endogenous cortico- terone production very shortly after its administration. This effect per- isted for a period of at least 8 hours. Because of these facts and since it has een found that there is a maximum elevation of adrenal and plasma corti- osterone approximately 2 hours after the administration of ACTH (7), a wo-hour lapse of time after injection of ACTH alone or in combination ith corticosterone was thought suitable to study the effects of these sub- ances. Thus it was surmised that if the animals were sacrificed 2 hours fter the injection of corticosterone one would expect a minimal adrenal orticosterone level at this time, a maximal level after ACTH administra- ion and a combination of effects when both substances were administered.

Experiment 2. This experiment was designed to ascertain how many units of ACTH would be required to just obtain a maximum response of the adrenal without "saturating" the animal with this substance. The dosage level for ACTH had to be adjusted also so that it would be expected to stimulate the adrenal glands maximally even when the endogenous output of ACTH would be repressed by administration of exogenous corticosterone. The results of the dose response experiment are shown in Table 2. The plateau of response to a single subcutaneous injection of ACTH begins at a dose level of 2 units. The amount of adrenal corticosterone can be related to the amount of ACTH administered only in a very approximate manner.

Experiments 3 and 4. Table 3 indicates that when 2 or 4 units of ACTH are injected in the presence of 5 mg. of corticosterone the level of adrenal corticosterone is less than when the same amount of ACTH is injected alone. The P value is not within the limits of acceptability when ACTH is injected at the 2 unit level. It is highly significant, however, at the 4 unit level ($P < 0.01$). When only corticosterone was injected the values are significantly lower than the control groups and confirm the results obtained in Experiment 1 (Table 1). Thus, from these results it would appear that corticosterone has an inhibitory effect upon its own production at the adrenal level. It is unlikely that the effects seen with the combination ACTH plus corticosterone can be attributed to the inhibition of endogenous ACTH by the corticosterone administered. It is not unreasonable to assume that the amount of circulating ACTH resulting from the injections of ACTH would be far greater than that amount which would have been produced by a fully activated hypophysis. Because the difference in the adrenal corticosterone level between the ACTH and ACTH plus corticosterone-treated animals was significant in only one of two cases (Experiment 4), an attempt was made to obtain more clear cut evidence using hypophysectomized animals. In this case also, the factor of endogenous production of ACTH would be eliminated.

Experiment 5. Due to the different experimental design when carrying

out similar studies as Experiment 4 in the hypophysectomized animal, and for reasons given in the experimental section, the results between Experiments 4 and 5 cannot be compared on an absolute basis. However, Table 4 shows that 0.17 ± 0.02 μg. of corticosterone per adrenal was found when the hypophysectomized rats were killed 14 hours after the last injection of ACTH as compared to 0.10 ± 0.03 μg. per adrenal found in the ACTH plus corticosterone group. The difference between these two groups of animals was highly significant ($P < 0.02$). In the ACTH group decapitated 2 hours after the last injection, the level of adrenal corticosterone was 0.38 ± 0.02 μg. per adrenal while the ACTH plus corticosterone group was 0.30 ± 0.04 μg. per adrenal. Although it would appear that there is no significant difference between the mean of the last two groups, the P (< 0.01) still indicates that the difference between the ACTH group (d) and the ACTH-corticosterone group (e) was highly significant. The explanation for this apparent paradox is due to the fact that in group (e) 2 values were very high (0.63, 0.56), whereas the 11 remaining ones were below 0.28 μg./adrenal. The control group at the time of sacrifice gave a mean value of 0.07 ± 0.01 μg. corticosterone/adrenal. These results support and extend those obtained in Experiments 3 and 4 and indicate that the production of corticosterone is to some extent regulated by the plasma level of the same compound. This inhibitory effect, therefore, would not appear to be mediated via the pituitary since it was obtained in normal (Experiments 3 and 4) as well as in the hypophysectomized (Experiment 5) rats. In the hypophysectomized rat the difference in adrenal corticosterone level between the ACTH and ACTH plus corticosterone treated groups varied greatly according to the time when the last injection was given. Thus, when the last injection was given 14 hours prior to sacrificing, the ACTH group was 70% higher in terms of adrenal corticosterone content than the ACTH plus corticosterone group. If the last injections were given 2 hours prior to sacrificing, the ACTH group was only 26% higher. These results suggest that the inhibitory effect of corticosterone on corticosteroid production at the adrenal level can be overcome by large doses of ACTH, or when ACTH is circulating at a high concentration. It is interesting to note also that the groups receiving ACTH had greater adrenal weights than the control group. This occurred independently of whether corticosterone was administered with ACTH or not. Therefore, it would appear that in the ACTH-corticosterone group the two main actions of the adrenocorticotropic hormone, a) stimulation of corticosteroidogenesis and b) trophic effect appear to be partially dissociated. This observation is supported by Stebbins' (11) findings which indicated that administration of corticoids does not decrease the adrenal weight of hypophysectomized rats.

Experiment 6. The results of this experiment are given in Table 5. The glands in the control group produced 3.73 ± 0.28 μg. of corticosterone per 100 mg. of wet adrenal tissue whereas glands incubated in the presence of

0.050 unit of ACTH produced 7.19 ±0.49 of corticosterone. The difference in the values was highly significant ($P < 0.001$). The stimulating effect of ACTH was not observed when corticosterone was added in the presence of ACTH. In group 3, 19.75 ±0.39 μg. of corticosterone per flask was recovered, a value not differing significantly from that found (20.24 ±0.34 μg./flask) in the corticosterone-ACTH group ($P > 0.30$). Since recoveries from medium alone following a one-hour incubation were 98.6 ±1.9% the low value 19.75 ±0.39 found for the corticosterone group can probably be attributed to incomplete recoveries. In agreement with the previous *in vivo* studies, these results indicate that corticosterone inhibits the action of ACTH by a direct action upon the adrenal gland.

Identical incubation experiments, carried out with smaller additions (5 and 10 μg.) of corticosterone, failed to show any inhibitory effect of the adrenal response to ACTH. The reason for the lack of an inhibitory effect at these concentration levels is unknown. It is conceivable that an optimal concentration of corticosterone must be present in the flasks before inhibition of ACTH action(s) occurs. If this is so it would preclude corticosterone acting as a competitive inhibitor of ACTH.

GENERAL DISCUSSION

The results reported here lend support to the theory that the amount of corticoid production in the normal or excited state is not governed solely by the hypophysis and the amount of ACTH which it releases. Thus, it is indicated that corticosterone, the main steroid hormone secreted by the rat adrenal, inhibits its own production by interfering with the action of ACTH at the adrenal level. This type of effect has also been reported with protein hormones which inhibit the glands which are involved in their manufacture. Thus, the exogenous administration of insulin results in an atrophy of the islets of Langerhans (12, 13) and the injection of ACTH to adrenalectomized rats produces a diminution in the ACTH content of the hypophysis (14, 15, 16). Birmingham and Kurlents (5) have also found that cortisol and corticosterone inhibit the action of ACTH *in vitro* and have suggested on the basis of this and other evidence that an inhibition of the pituitary adrenal system by adrenal cortical steroids may occur at the level of the adrenal cortex. It is interesting to note also that the work of Langecker and Lurie (4) support the above possibilities. They found that the adrenals of hypophysectomized rats pre-treated with cortisone had a much lower response to injected ACTH than those rats receiving no cortisone as measured by the ascorbic acid depletion test.

It is difficult at present to know whether the results obtained represent those which can possibly occur under normal physiological conditions. Although the amount of corticosterone injected in Experiments 3 and 4 appears large, the concentration of plasma corticosterone at the two-hour period was only twice that which was found when 2 units of ACTH were

administered (101.5 μg. % versus 47.7 μg. %) (7). Thus, the relatively large subcutaneous injections of corticosterone do not elevate the circulating blood corticosterone to levels which are greatly abnormal for any one period of time.

Further studies will be required to investigate whether different concentrations of plasma corticosterone will give different degrees of inhibition at the adrenal level.

Any attempt to predict at which point or step corticosterone inhibits ACTH action would be speculative. Experiments designed to investigate this problem will have to be run with the view in mind of answering the question as to whether corticosterone inhibits the primary step(s) which ACTH activates (19) and/or whether it inhibits at different step(s) in the corticosteroidogenic chain for, *e.g.*, pregnenolone to desoxycorticosterone.

Addendum

Since submission of the manuscript additional *in vitro* experiments were carried out by incubating corticosterone and cortisol in the presence of ACTH. We have not been able to substantiate the results of the *in vitro* experiment reported above when the same concentration of steroids and ACTH were used in the incubations. However, *in vivo* experiments carried out with cortisol and cortisone are in complete accord with the reported results and conclusions.

REFERENCES

1. Ingle, D. J., G. M. Higgins and E. C. Kendall: *Anat. Record* **71:** 363. 1938.
2. Guillemin, R., W. R. Hearn, W. R. Cheek and D. E. Housholder: *Endocrinology* **60:** 488. 1957.
3. Urquhart, J., F. E. Yates and A. L. Herbst: *Endocrinology* **64:** 816. 1959.
4. Langecker, H. and R. Laurie: *Acta Endocrinologica* **25:** 54. 1957.
5. Birmingham, M. K. and E. Kurlents: *Endocrinology* **62:** 74. 1958.
6. Silber, R. H., R. D. Busch and R. Oslapas: *Clin. Chem.* **4:** 278. 1958.
7. Moncloa, F., F. G. Péron and R. I. Dorfman: *Endocrinology* **65:** 717. 1959.
8. Péron, F. G. and R. I. Dorfman: *Endocrinology* **64:** 431. 1959.
9. Romanoff, E. B. and C. A. Hunt: *Am. J. Physiol.* **179:** 15. 1954.
10. Saffran, M. and A. V. Schally: *Endocrinology* **56:** 523. 1955.
11. Stebbins, R. B.: *Endocrinology* **49:** 671. 1951.
12. Latta, J. S. and H. T. Harvey: *Anat. Rec.* **82:** 281. 1942.
13. Mirsky, I. A., N. Nelson, S. Elgart and I. Grayman: *Science* **95:** 583. 1942.
14. Gemzell, C. A. and F. Heijkenskjold: *Acta Endocrinologica* **24:** 249. 1957.
15. Kitay, J. I., D. A. Holub and J. W. Jailer: *Proc. Soc. Exptl. Biol. & Med.* **97:** 165. 1958.
16. Ibid: *Endocrinology* **64:** 475. 1959.
17. Schally, A. V., M. Saffran and B. Zimmermann: *Biochem. J.* **70:** 97. 1958.
18. Saffran, M., A. V. Schally and B. G. Benfey: *Endocrinology* **57:** 439. 1955.
19. Péron, F. G. and S. Koritz. *J. Biol. Chem.* In press.

PRODUCTION AND CATABOLISM OF CORTISOL FOLLOWING THE ADMINISTRATION OF THYROXINE ANALOGS[1,2,3]

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ABSTRACT

Permanent cannulas were placed in the adrenal veins of dogs and minute cortisol output was measured prior to and following injections of triiodothyroacetic acid, triiodothyronine and thyroxine. Cortisol output was increased within 30 to 120 minutes after the injection of the thyroxine analogs, but not after thyroxine, to the same extent as that found in control animals given corticotropin. The adrenal response to thyroxine analogs could not be obtained in hypophysectomized dogs. Infused cortisol disappeared from the plasma at a more rapid rate in dogs pretreated with triiodothyronine than in controls. It is suggested that the rate of peripheral steroid metabolism is increased due to the thyroxine analogs, which in turn stimulates the release of corticotropin.

WHEN cortisol was infused into patients with thyrotoxicosis the turnover rate of the hormone was accelerated on the basis of the calculated removal of the steroid from the plasma (1). The study described in this paper was undertaken to clarify the direct effect of the thyroid hormone or its analogs on the production of cortisol, the principal glycocorticoid secreted by the canine adrenal cortex (2).

MATERIALS AND METHODS

Adult mongrel dogs ranging from 9 to 24 Kg. in weight were used. All were anesthetized with pentobarbital sodium (30 mg./Kg.) before being subjected to experimental

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[2] The data contained in this paper is part of a thesis submitted by the senior author to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[3] This work was presented in part on the program of the 38th Annual Meeting of the Endocrine Society, New York, N. Y., June, 1957.

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procedures. A permanent polyethylene cannula was placed in the right lumbo-adrenal vein of each of 26 dogs, according to the technique described by Hume and Nelson (3). This preparation permitted intermittent collection of the total venous effluent of the right adrenal gland. Adrenal venous blood was collected for 5-minute periods at varying intervals; each cannula being filled with 4% heparin in physiological saline solution between the periods of collection. Experiments were carried out 18 to 24 hours after the cannula had been inserted. Peripheral blood specimens, obtained from the femoral vein by means of a small gauge polyethylene catheter, and the samples of adrenal vein blood were analyzed for cortisol by a modification of the method of Silber and Porter (4) as described by Wu and Mason (5). This method is specific for steroids with the 17,21-dihydroxy-20-keto configuration, including cortisol, cortisone, compound S, and their dihydro- and tetrahydro-derivatives. Within this group, only cortisol exists in canine plasma in significant concentrations.

Solutions of 3:5:3′-L-triiodothyronine, 3:5:3′-L-triiodothyroacetic acid and L-thyroxine[7] were prepared as follows: 40 mg. of each was dissolved in 13 ml. of 0.01 N sodium hydroxide and 900 mg. of sodium chloride was added. These solutions were diluted to a final volume of 100 ml. with cold, freshly distilled water and autoclaved at 10 pounds pressure for 30 minutes; 0.1 ml. of these solutions contained 40 μg. of each of the thyroid hormones. The pH of the solutions was approximately 8.0.

A solution containing no hormone was prepared in the same way and was designated as "vehicle" for purposes of control. All solutions were renewed after 4 days.

EXPERIMENTAL PROCEDURE

After collection of a sample of adrenal venous blood, each of 10 dogs received 4 mg. of triiodothyroacetic acid rapidly injected intravenously. Adrenal venous effluent was collected at 30, 60, 120, and 180 minutes following the injection. The specimens were then analyzed for cortisol, and the output of cortisol from the right adrenal gland was calculated. After control specimens were obtained, an injection of 0.8 mg. of L-triiodothyronine was given each of three dogs; a dose of 4 mg. of L-thyroxine was injected into three others; and 10 ml. of vehicle was rapidly injected into two dogs. The collection and treatment of specimens of adrenal venous blood have been described above. Twenty-five U.S.P. units of corticotropin (Upjohn, Sterile Corticotropin Injection) dissolved in 10 ml. of normal saline was rapidly injected intravenously and serial blood specimens were collected from the right adrenal at the same intervals as those following the injection of the thyroid hormone analogs.

Five animals with permanent adrenal vein cannulas underwent hypophysectomy through a transtemporal approach. Experiments on these dogs were carried out within 3 to 6 hours following hypophysectomy. Four mg. of L-triiodothyroacetic acid was injected intravenously into each of the hypophysectomized dogs after a control specimen has been obtained. Subsequent samples of adrenal venous blood were obtained at 30, 60, 120, and 180 minutes following the injection.

In order to determine the rate of removal of exogenous cortisol from the plasma, an intravenous injection of cortisol as the succinate ester dissolved in 2 ml. of distilled water (Upjohn, Hydrocortisone Sodium Succinate) was given in a dosage of 5 mg./Kg. to 10 normal anesthetized dogs and to five dogs that had received intravenous injections of L-triiodothyronine in a dose of 10 μg./Kg. at 24 and 6 hours prior to the injection of cortisol.

[7] The L-triiodothyronine, L-triiodothyroacetic acid and L-thyroxine were generously supplied by Smith, Kline and French Laboratories through the courtesy of Mr. John Clipson.

Samples of peripheral (femoral) venous blood were obtained immediately preceding the administration of the cortisol sodium succinate and at 30, 60, 90, and 120 minutes thereafter. Plasma cortisol concentrations were determined as described. The sodium succinate ester of cortisol was employed because it is highly soluble and can be administered in a small volume of aqueous diluent in a few seconds. The ester is not extracted from plasma by methylene chloride which is the solvent used in the determination of plasma cortisol (6).

RESULTS

Adrenal secretory response to thyroxine analogs, L-*thyroxine, and corticotropin.* The mean outputs of cortisol from the right adrenal gland in response to these stimuli are shown in Table 1. The results are tabulated as mean values for the control period and as mean maximum values reached

TABLE 1. CORTISOL OUTPUT FROM THE RIGHT ADRENAL GLAND IN RESPONSE TO ANALOGS OF THYROXINE

Test material	Dose (mg.)	No. of dogs	Pituitary status	Cortisol output in μg./min.	
				Mean control levels	Mean maximum values after test material
L-triiodothyroacetic acid	4.0	10	Intact	1.1±0.7*	10.9±4.2
L-triiodothyroacetic acid	4.0	5	Hypox.†	0.4±0.3	0.6±0.1
L-triiodothyronine	0.8	3	Intact	0.9	9.7±2.8
L-thyroxine	4.0	3	Intact	0.3	0.7
Corticotropin	25 units	3	Intact	0.5	11.7
Vehicle	—	2	Intact	0.5	0.8

* Standard deviation.
† Hypophysectomized.

during the test period. The basal output of cortisol averaged less than 1 μg./min.

No significant change in adrenal secretory activity occurred following injection of the vehicle or L-thyroxine. Injection of triiodothyroacetic acid failed to elicit an adrenal secretory response in hypophysectomized dogs. The degree of stimulation of cortisol secretion following triiodothyronine and triiodothyroacetic acid is comparable to that obtained following intravenous corticotropin.

Rate of disappearance from the plasma of cortisol in dogs pretreated with triiodothyronine. In Table 2 are presented the mean concentrations of plasma cortisol in dogs pretreated with triiodothyronine and in controls before and after an intravenous injection of cortisol as the succinate ester. Although the ranges of cortisol levels are considerable, the values for each individual animal plotted on semilogarithmic paper are linear and the calculated standard errors of the differences (S_D) of the means of the control and test groups indicate that the differences are highly significant. From the data obtained from each individual dog the biological half-life

TABLE 2. THE DISAPPEARANCE OF CORTISOL FROM THE PLASMA IN DOGS PRETREATED WITH TRIIODOTHYRONINE FOLLOWING AN INJECTION OF CORTISOL (5 MG./KG.) AS THE SUCCINATE ESTER

	No. of dogs	Mean plasma cortisol concentration in μg./100 ml.					Mean biological half-life of infused cortisol in plasma (in minutes)
		Prior to exogenous cortisol	Minutes after injection of cortisol 21-hemisuccinate				
			30	60	90	120	
Controls	10	7 (0–12)*	128 (100–193)	91 (75–146)	66 (41–126)	45 (25–91)	56.4
Pretreated with L-triiodothyronine	5	9 (6–16)	142 (118–168)	56 (41– 72)	32 (28– 36)	18 (14–25)	31.3
S_D**		±1.33	±12.3	±9.2	±8.0	±6.8	p = <.001

* Range.
** Standard error of the difference of means of control and test groups.

of infused cortisol was calculated by the method of the least squares. The mean biological half-lives are shown in Table 2. The mean biological half-lives are based on the average slope of individual cortisol levels plotted on semilogarithmic paper. The "t" value derived was 4.99 which is well over the 99% confidence limits. The rate of disappearance of infused cortisol is much more rapid in animals pretreated with triiodothyronine. The biological half-time in the pretreated group was only 55% of the control.

DISCUSSION

These experiments show that adrenal secretion of cortisol is increased shortly after an intravenous injection of active analogs of thyroxine. The magnitude of adrenal secretory response is similar to that observed following a large dose of corticotropin. An intact pituitary is essential for thyroxine analogs to augment cortisol production. The lack of adrenal response to thyroxine may be due to the latent period involved in thyroxine activity, since deiodination of thyroxine by the tissues to a more active form has been postulated.

The biolgical half-life of injected cortisol is shortened in animals pretreated with triiodothyronine. This phenomenon has been observed in humans with thyrotoxicosis and is thought to represent increased catabolism of the steroid by the liver (1). *In vitro* studies by Yates *et al.* (7) have shown accelerated metabolic transformation (Ring A reduction) of cortisone by liver from rats pretreated with triiodothyronine. McGuire *et al.* (8) have demonstrated that the rate of Ring A reduction of steroids is dependent upon available TPNH, which regularly increases in rats pretreated with triiodothyronine. It is possible that accelerated enzymatic reduction of circulating cortisol provides the stimulus to the release of

corticotropin following an injection of the active analogs of thyroxine in the dog.

REFERENCES

1. Peterson R. E.: *J. Clin. Invest.* **37:** 736. 1958.
2. Farrell, G. L.: *Proc. Soc. Exper. Biol. and Med.* **86:** 338. 1954.
3. Hume, D. M. and D. H. Nelson: *Surg. Forum* **5:** 568. 1955.
4. Silber, R. H. and C. C. Porter: *J. Biol. Chem.* **210:** 923. 1954.
5. Wu, C. and H. L. Mason: Proc. of Staff Meet. Mayo Clinic **33:** 627. 1958.
6. Melby, J. C. and W. W. Spink: *J. Clin. Invest.* **37:** 1791. 1958.
7. Yates, F. E., J. Urquhart and A. L. Herbst: *Am. J. Physiol.* **195:** 373. 1958.
8. McGuire, J. S. and G. M. Tomkins: *J. Biol. Chem.* **234:** 791. 1959.

SOME BIOLOGICAL PROPERTIES OF A PERHYDROPHENANTHRENE DERIVATIVE[1]

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ABSTRACT

The compound Ro 2-7239 (2-acetyl-7-oxo-1,2,3,4,4a,4b,5,6,7,9,10,10a-dodecahydrophenanthrene) has been demonstrated to possess androgenic and anti-androgenic activity when tested on the chick's comb and the seminal vesicles, prostate, and levator ani muscle of the castrated rat. A rough estimate would assign a potency of 1/100th the relative activity on the basis of the chick comb and 1/400th on the basis of the rat accessories when compared to testosterone. Quite unlike testosterone Ro 2-7239 produces a shallow dose-response curve.

Ro 2-7239 possesses no demonstrable estrogenic activity either by subcutaneous injection (less than 1/16,000th of estrone) or by gavage (less than 1/300th of estrone). Neither could anti-estrogenic activity be demonstrated. This phenanthrene derivative, when administered orally or by subcutaneous injection, inhibited the formation and/or release of pituitary gonadotropin as judged by the ovarian weight of an intact female in parabiosis with a castrated male rat.

PREVIOUS studies have clearly indicated that 2-acetyl-7-oxo-1,2,3,4,-4a,4b,5,6,7,9,10,10a-dodecahydrophenanthrene (Ro 2-7239) possesses both androgenic activity in the chick (1) and anti-androgenic properties in the rat (2) and chick (3). This communication deals with the androgenic properties of this compound both in the chick, as previously reported, and in the castrated rat, which is described here for the first time, in addition to other biological properties including pituitary gonadotropin inhibition.

METHODS AND MATERIALS

White leghorn chicks were obtained from Hall Bros. Hatchery in Wallingford, Conn., and arrived in the laboratory within one day of hatching. The various test materials were admixed with food, injected in an aqueous suspension consisting of sodium chloride (0.9%), polysorbate 80 (0.4%), carboxymethylcellulose (0.5%), and benzyl alcohol (0.9%), or inuncted directly on the comb in a sesame oil solution. The detailed procedures for each experiment are recorded in the tables and in each instance comb and body weights were determined 24 hours after last treatment.

Male albino rats were obtained from The Charles River Breeding laboratories and arrived in the laboratory at 21 days of age and were used 4 to 7 days later. The test

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compounds, suspended in the same aqueous vehicle as used for the chick experiments, were injected subcutaneously. If two compounds were administered simultaneously by injection, separate sites were used to avoid problems of interaction or absorption delays at the site.

Standard histological procedures were used in preparing the tissue for microscopic evaluation. The freshly removed tissues were coded to disguise their identity, fixed in 10% formalin, dehydrated, imbedded in paraffin, and serial sections cut at 4 microns. The sections were stained with Delafield's hematoxylin, counterstained with eosin, and mounted in balsam.

EXPERIMENTAL

Chick Anti-Androgen Studies

The fact that Ro 2-7239 inuncted directly to the chick comb can inhibit the stimulating action of subcutaneously administered testosterone has

TABLE 1. CHICK ANTI-ANDROGEN ASSAY

Conditions of Assay: 2-Day old male chicks were fed Δ^4-androstene-3,17-dione in food (80 mg./kilo). Ro 2-7239 (R) was inuncted in sesame oil (0.05 ml./day) for seven days.

Material administered	Total dose, mg.	No. of chicks	Mean comb ratio ± S.E.**
0	0	24	1.31 ± 0.054
R	0.25	19	0.98 ± 0.053
	0.5	20	1.02 ± 0.053
	1.0	20	0.95 ± 0.062
	2.0	20	0.77 ± 0.047

** Comb Ratio = $\frac{\text{mg. of comb}}{\text{gm. of body weight}}$.

S.E. = Standard Error.

been previously described (3). Similar studies are now reported for Δ^4-androstene-3,17-dione and methyltestosterone. When 0.25 to 1.0 mg. of Ro 2-7239 was inuncted on the combs of chicks fed Δ^4-androstene-3,17-dione, statistically significant reductions in comb size were found. The dose of 2.0 mg. produced a comb ratio of 0.77 ± 0.047. Since a group of 20 chicks of the same age and sex, receiving only the vehicle, had a comb ratio of 0.43 ± 0.019 and since the androgen fed chicks had a comb ratio of 1.31 ± 0.054, the per cent inhibition with this higher dose was actually 62% (Table 1). No significant differences were observed in mean body weights for any of various groups of chicks.

When methyltestosterone admixed with the food was the stimulating androgen, inhibition of comb growth was demonstrated when 2 mg. of Ro 2-7239 was inuncted on the comb (Table 2).

Norethisterone (17α-ethynyl-19-nortestosterone) is known to inhibit the action of testosterone on the chick's comb (1, 7) and the potency of this inhibitor is of the order of that found for Ro 2-7239. Various combinations of the two agents were studied (Table 3) in an effort to intensify the inhibition. No intensification could be demonstrated, even when maximum doses

TABLE 2. CHICK ANTI-ANDROGEN ASSAY

Conditions of Assay: 2-Day old male chicks were fed methyltestosterone in food (10 mg./kilo). Ro 2-7239 (R) was inuncted in sesame oil (0.05 ml./day) for 7 days.

Material administered	Total dose, mg.	No. of chicks	Mean comb ratio ± S. E.
0	0	14	0.55±0.027
R	0.5	14	0.53±0.040
	1.0	14	0.49±0.028
	2.0	13	0.42±0.025

were inuncted simultaneously. At the 1 mg. dose level Ro 2-7239 alone gave a comb ratio of 0.81 ±0.038, as compared to 1.29 ±0.052 and 1.27 ±0.052 for the oil inuncted chick combs. At the same dose level norethisterone gave a comb ratio of 0.82 ±0.032. The inunction of both agents at the same dose level gave a comb ratio of 0.90 ±0.032.

Rat Anti-Androgen Studies

Previous studies by Randall and Selitto (2) established the anti-andrc-genic activity of Ro 2-7239 in castrated rats stimulated with testosterone. These results are confirmed and extended in this report (Table 4). Ro 2-7239 inhibited the action of Δ^4-androstene-3,17-dione on both the seminal vesicles and prostate but the inhibition of this androgenic action on the levator ani was somewhat variable.

At the 50 mg. dose of Ro 2-7239 minimum significant decreases in levator ani weight were found in two of the three experiments, but in-

TABLE 3. INHIBITORY ACTION OF RO 2-7239 AND NORETHISTERONE ALONE AND IN COMBINATION IN TESTOSTERONE STIMULATED CHICKS

(7-Day test—all chicks received 0.5 mg. of testosterone enanthate by subcutaneous injection on the first day).

Ro 2-7239 inuncted, mg.	Norethisterone inuncted, mg.	No. of chicks	Mean comb ratio ±S.E.
0	0	25	1.29±0.052
0	0	23	1.27±0.052
0	0.125	20	1.02±0.056
0	0.250	19	1.16±0.070
0	0.5	19	0.92±0.036
0	1.0	21	0.82±0.032
0.032	0	18	1.07±0.054
0.063	0	19	0.92±0.042
0.125	0	19	1.00±0.043
0.250	0	20	0.85±0.042
0.5	0	20	0.84±0.035
1.0	0	18	0.81±0.038
0.032	0.125	19	0.84±0.040
0.125	0.125	19	0.83±0.037
0.25	0.125	20	0.93±0.050
0.5	0.125	19	0.94±0.050
1.0	0.125	19	0.83±0.043
0.063	0.25	19	0.91±0.044
0.5	0.5	18	0.90±0.036
1.0	1.0	19	0.90±0.032

TABLE 4. RAT ANTI-ANDROGEN ASSAY

Conditions of Assay: 26 to 27 day old castrated rats were injected subcutaneously with 0.5 ml./day for 7 days. Compounds were suspended in aqueous medium.

T = Testosterone
A = Δ^4-Androstene-3,17-dione
R = Ro 2-7239

Exp. No.	Material administered	Total dose, mg.	No. of rats	Mean tissue ratio ± S.E.		
				Seminal vesicle	Prostate	Levator ani
A	0	0	7	0.05±0.005	0.07±0.007	0.24±0.031
	A	8	6	0.50±0.049	0.72±0.052	0.46±0.027
	A R	8 50	7	0.30±0.020	0.42±0.026	0.48±0.053
	T	2	7	0.94±0.058	0.96±0.072	0.67±0.045
	T R	2 50	7	0.68±0.053	0.65±0.031	0.57±0.026
B	0	0	10	0.07±0.004	0.08±0.009	0.23±0.012
	A	8	10	0.37±0.051	1.01±0.066	0.46±0.066
	A R	8 50	10	0.34±0.027	0.98±0.054	0.39±0.025
	A R	8 150	10	0.25±0.011	0.67±0.062	0.45±0.024
	T	2	8	0.90±0.100	1.19±0.061	0.63±0.34
	T R	2 50	9	0.81±0.087	1.00±0.041	0.59±0.037
	T R	2 150	10	0.67±0.087	0.93±0.041	0.57±0.034
C	0	0	8	0.11±0.004	0.14±0.015	0.29±0.009
	A	8	9	0.80±0.075	1.23±0.111	0.56±0.036
	A R	8 50	10	0.50±0.046	1.21±0.108	0.48±0.025
	A R	8 150	10	0.40±0.037	0.85±0.082	0.56±0.023
	T	2	10	1.11±0.080	1.40±0.088	0.74±0.043
	T R	2 50	10	0.94±0.056	1.09±0.059	0.67±0.021
	T R	2 150	10	0.83±0.057	0.90±0.065	0.65±0.028
D	0	0	15	0.14±0.007	0.16±0.10	0.21±0.015
	T	2.4	15	1.96±0.10	1.35±0.12	0.55±0.005
	T R	2.4 0.5	14	1.61±0.12	1.40±0.14	0.57±0.031
	T R	2.4 1.0	15	1.32±0.099	1.15±0.076	0.56±0.034
	T R	2. 4.0	14	1.15±0.062	1.04±0.073	0.49±0.027
	T R	2.4 10.0	14	1.44±0.066	1.26±0.056	0.58±0.032

creasing the dosage to 150 mg. caused a reversal of effect; that is, the weight of the levator ani was the same as when the gland was only stimulated with the androgen. In Experiment B the levator ani ratio was 0.46 $\pm$0.066 when Δ^4-androstene-3,17-dione alone was injected. At a dose of 50 mg. of Ro 2-7239 in addition to the androgen, the ratio decreased to 0.39 $\pm$0.025 and increased again to 0.45 $\pm$0.024 when 150 mg. of the phenanthrene derivative was employed.

Experiment D is unique since it tends to illustrate the fact that there is an optimum dose for anti-androgenic activity of Ro 2-7239. In this experiment doses of Ro 2-7239 from 0.5 mg. to 4.0 mg. showed a progressively greater anti-androgenic effect on all three indicators. When the

TABLE 5. RAT ANTI-ANDROGEN ASSAY

Conditions of Assay: 26-Day old albino rats were injected subcutaneously with testosterone (T) in 0.5 ml./day for 7 days and Ro 2-7239 (R) was gavaged in 0.5 ml./day for 7 days.

Material administered	Total dose, mg.	No. of rats	Mean tissue ratio $\pm$ S.E.		
			Seminal vesicle	Prostate	Levator ani
0	0	8	0.10 $\pm$ 0.007	0.16 $\pm$ 0.009	0.21 $\pm$ 0.013
T	2.4	9	1.03 $\pm$ 0.075	1.24 $\pm$ 0.082	0.40 $\pm$ 0.017
T R	2.4 100	10	0.97 $\pm$ 0.087	0.98 $\pm$ 0.062	0.43 $\pm$ 0.017

dose was increased to 10 mg. the inhibitory effect on the levator ani and prostate was no longer observed and the anti-androgenic action on the seminal vesicles was significantly lessened. No significant differences could be detected in the final body weights of the castrated rats treated with testosterone, Δ^4-androstene-3,17-dione, or these androgens plus the inhibitor Ro 2-7239.

The anti-androgenic activity of Ro 2-7239 was assessed by gavage in a single experiment. The compound, at least in this preliminary study, showed a lower activity by the oral route since at a total dose of 100 mg. significant anti-androgenic activity was found only for the stimulated prostate gland (Table 5).

Androgen Assay in Chicks

The phenanthrene derivative produced an increase in the weight of the chick's comb by direct inunction and by subcutaneous injection in one experiment. A preliminary report of the androgenicity of Ro 2-7239 by direct comb inunction has been published (3). In Table 6 additional experiments are presented to prove this point. In Experiments E through J (Table 6) the vehicle was ethanol with the exception of one experiment, I, where sesame oil was employed. Statistically significant increases in comb ratio were found in each experiment and no significant changes were

TABLE 6. CHICK COMB ANDROGEN ASSAY BY INUNCTION

Conditions of Assay: Testosterone (T) and Ro 2-7239 (R) inuncted once daily for 7 days 0.05 ml. of vehicle. Male chicks 1 to 2 days of age on first day of experiment.

Exp. No.	Vehicle	Material administered	Total dose, μg.	No. of chicks	Mean comb ratio ±S.E.
E	Ethanol	0	0	21	0.43±0.015
		T	1	19	0.65±0.023
			4	19	0.71±0.030
			16	19	0.91±0.044
		R	1	20	0.54±0.021
			10	19	0.52±0.019
			100	17	0.65±0.034
			1000	20	0.83±0.022
F	Ethanol	0	0	20	0.32±0.016
		T	1	20	0.39±0.015
			4	20	0.56±0.019
			16	19	0.70±0.033
		R	1	20	0.34±0.019
			10	20	0.33±0.009
			100	20	0.40±0.022
			1000	19	0.44±0.021
G	Ethanol	0	0	15	0.33±0.018
		T	1	14	0.43±0.019
			4	15	0.47±0.027
			16	12	0.68±0.047
		R	10	12	0.42±0.018
			100	12	0.47±0.024
			500	13	0.60±0.036
H	Ethanol	0	0	18	0.39±0.021
		T	3	14	0.48±0.028
			9	13	0.59±0.038
			27	13	0.97±0.052
		R	1	14	0.43±0.024
			50	13	0.50±0.029
			1000	12	0.56±0.034
I	Sesame Oil	0	0	31	0.37±0.013
		T	3	30	0.49±0.020
			9	30	0.51±0.018
			27	27	0.63±0.034
		R	1	29	0.35±0.013
			50	29	0.38±0.011
			250	30	0.46±0.015
			1000	30	0.46±0.015
J	Ethanol	0	0	13	0.26±0.011
		T	1	10	0.33±0.014
			3	8	0.37±0.029
			9	10	0.69±0.046
			27	9	0.86±0.070
		R	250	9	0.46±0.028
			500	10	0.44±0.025
			1000	10	0.46±0.028
			2000	10	0.57±0.032

observed in body weights of the chicks. At 10 μg. two of three trials were positive, at 50 μg. one of two trials was positive, but at 100 μg. three of three trials resulted in statistically significant comb increments. Every trial was positive when 100 μg. or more of Ro 2-7239 was inuncted. By comparison, about one microgram of testosterone usually produces a significant increment.

Although 100 μg. of Ro 2-7239 or more produced a significant minimum comb stimulation, increasing the dosage by a factor of 10 did not consist-

TABLE 7. CHICK ANDROGEN ASSAY BY SUBCUTANEOUS INJECTION

Testosterone (T) and Ro 2-7239 (R) were injected subcutaneously once daily in 0.1 ml. aqueous suspension for 7 days.

Exp. No.	No. of days	Material injected	Total dose, mg.	No. of chicks	Mean comb ratio ±S.E.
K	14	0	0	22	0.57±0.041
		R	1	19	0.71±0.060
			3	19	0.91±0.072
L	7	0	0	12	0.28±0.012
		T	0.04	11	0.33±0.010
			0.08	11	0.44±0.019
			0.16	10	0.46±0.026
		R	0.1	11	0.27±0.007
			0.5	9	0.28±0.018
			2.5	11	0.31±0.017
M	7	0	0	12	0.33±0.010
		T	0.005	10	0.37±0.024
			0.015	10	0.41±0.031
			0.045	10	0.44±0.023
			0.135	10	0.57±0.039
		R	0.005	10	0.31±0.015
			0.030	10	0.36±0.016
			0.180	10	0.36±0.018
			1.0	10	0.34±0.018
			6.0	10	0.34±0.024

ently produce a significantly greater comb response. This is in contrast to observations with most steroidal androgens. At the 500 to 1000 μg. dose levels increases in comb ratio of 27% to 82% were found.

The data in Table 7, Experiment K, indicate that Ro 2-7239 is an active androgen when administered by injection. This experiment was done in an attempt to inhibit the endogenous androgens produced by chick's adrenals and/or gonads. Since it is known that the comb increases faster than the body weight between the 7th and 14th day, it was expected that administration of Ro 2-7239 would result in a lowered comb size in the treated groups but this was not the case; on the contrary, a stimulation of the comb was actually found. A total dose of one milligram produced a small

but significant increase in comb ratio from 0.57 ±0.041 to 0.71 ±0.062. At the total dose level of 3 mg. an increase in comb ratio of 63% was found.

In two 7-day experiments (L and M) it was not possible to demonstrate a significant increase in comb weight even when a total dose of 6 mg. of Ro 2-7239 was used.

The histological sections of control, testosterone and Ro 2-7239 treated chick combs were evaluated as unknowns. It was possible to distinguish between combs of vehicle control and treated animals, but not possible to distinguish between the combs of testosterone and Ro 2-7239 treated chicks. Both compounds caused an increase in the width of the connective tissue layer of the comb. The increase in width occurred peripherally under the epithelium and along the base of the comb. The dense, closely packed connective tissue in the vehicle control comb was replaced with diffuse connective tissue, few collagen fibers, and many vacuoles. In addition, the developing serrated dorsal aspect of the stimulated comb was evident as compared to the flat dorsal surface of control combs.

Androgenic Assay in Castrated Rats

Subcutaneous injection of Ro 2-7239 to the castrated rat produced weight increases in the seminal vesicles, prostate, and levator ani muscle without significant changes in body weight (Table 8). At the 100 mg. dose level increases in levator ani ratios of 90%, 56% and 83% wer found for Experiments N, O and P, respectively, and even greater percentage weight increases for the prostate. In Experiments O and P significant increases were found for the seminal vesicles, but not for Experiment N.

The structure of the normal rat prostate and the effects of testosterone on rat prostates have been described by Moore (4). It was possible to distinguish clearly between the prostate glands of vehicle control rats and those treated with either testosterone or Ro 2-7239 and further possible to distinguish histologically between these glands from testosterone and Ro 2-7239 treated rats. The Ro 2-7239 treated rats had prostates which presented a histological picture somewhere between that of the vehicle control and testosterone animals. In the stimulated glands the epithelial cells lining the acini tended to be more columnar, had darker staining nuclei and the cytoplasm above the nucleus had clear globular areas as opposed to the glands from vehicle control rats. There was some increase in folding of the alveoli in glands from Ro 2-7239 treated rats but not as extensive as appeared under testosterone treatment.

Moore (5) described the normal structure of rat seminal vesicles and the stimulating effects of testosterone. As in the prostate, it was possible to distinguish histologically the glands of testosterone and Ro 2-7239 treated animals from those of vehicle control rats and to distinguish between glands stimulated with testosterone and Ro 2-7239 treated. The seminal vesicles from rats treated with the latter compound were inter-

mediate and their histological pattern between vehicle control and testosterone stimulated glands in that the epithelial cells were more columnar (as with testosterone treatment) but the lumen of the folded tubule tended to be the same diameter as the vehicle control glands and not greatly increased in diameter as with testosterone stimulation. Testosterone treatment resulted in a decrease in the connective tissue stroma around the lumen, whereas the amount of connective tissue stroma in Ro 2-7239

TABLE 8. CASTRATE RAT ANDROGEN ASSAY OF Ro 2-7239 (R) COMPARED TO TESTOSTERONE (T) BY SUBCUTANEOUS INJECTION

Conditions of Assay: Rats castrated at 26–28 days of age and injected subcutaneously once daily with 0.5 ml. of aqueous suspension of compounds for 10 days.

Exp. No.	Material administered	Total dose, mg.	No. of rats	Final mean body wt., gm.	Mean tissue ratio ± S.E.		
					Seminal vesicle	Prostate	Levator ani
N	0	0	10	121	0.09±0.006	0.10±0.008	0.21±0.013
	T	0.25	10	137	0.14±0.011	0.31±0.021	0.27±0.015
		1	10	140	0.63±0.096	0.79±0.057	0.51±0.038
	R	1	10	130	0.08±0.005	0.10±0.008	0.30±0.014
		10	10	131	0.08±0.003	0.08±0.008	0.26±0.020
		100	10	130	0.11±0.008	0.27±0.020	0.40±0.021
O	0	0	17	118	0.07±0.002	0.10±0.005	0.25±0.009
	T	0.5	17	121	0.50±0.058	0.60±0.047	0.50±0.024
		1	15	120	0.53±0.030	0.70±0.033	0.51±0.020
		2	15	124	0.64±0.075	0.72±0.050	0.51±0.020
	R	1	15	123	0.08±0.004	0.10±0.006	0.25±0.007
		10	16	117	0.09±0.005	0.11±0.006	0.30±0.009
		50	15	113	0.11±0.007	0.18±0.020	0.35±0.015
		100	16	113	0.13±0.009	0.23±0.020	0.39±0.020
P	0	0	5	93	0.07±0.005	0.13±0.018	0.23±0.017
	T	2.4	9	90	1.10±0.079	1.31±0.038	0.69±0.053
	R	20	8	93	0.08±0.006	0.14±0.013	0.25±0.006
		100	6	92	0.12±0.006	0.38±0.027	0.42±0.027

stimulated seminal vesicles appeared to be identical with that in the control tissue.

Histological studies of the levator ani revealed a distinct difference in the skeletal muscle of stimulated animals both with testosterone and Ro 2-7239. It was not possible to distinguish between testosterone and Ro 2-7239 stimulated levator ani muscles. The striated muscle fibers of the stimulated animals were enlarged and appeared as distinct undulating lines. They also tended to be less closely packed together than the striated muscle fibers of the control levator ani.

When Ro 2-7239 was administered by gavage minimal stimulation was observed (Table 9). At the total dose levels of 3 and 9 mg. the phenanthrene

TABLE 9. ANDROGEN AND ANABOLIC ASSAY

Conditions of Assay: White albino rats 28-day old males (Charles River). Castrated and eated by gavage (0.5 ml./day) for 10 days.

Material administered	Total dose, mg.	No. of animals	Mean body wt., gm.	Seminal vesicle, mean ratio ±S.E.	Prostate, mean ratio ±S.E.	Levator ani, mean ratio ±S.E.
0	0	9	110	0.08±0.005	0.11±0.007	0.22±0.011
Halotestin	0.15	8	106	0.11±0.012	0.12±0.012	0.22±0.013
	0.6	9	104	0.17±0.014	0.23±0.029	0.25±0.015
	2.4	10	108	0.32±0.049	0.35±0.025	0.33±0.020
Ro 2-7239	1.0	10	111	0.09±0.009	0.14±0.028	0.18±0.013
	3.0	10	111	0.14±0.014	0.18±0.025	0.24±0.021
	9.0	9	108	0.14±0.013	0.16±0.014	0.26±0.012
	27.0	9	101	0.10±0.005	0.13±0.013	0.19±0.019
	81.0	9	108	0.10±0.009	0.14±0.014	0.21±0.012

derivative produced statistically significant increments in the levator ani muscle of 25% and 42%, respectively. Increasing the dosage to 27 and 81 mg., respectively, abolished the stimulation. Similar results were observed for the seminal vesicles and prostate. Doses of 3 and 9 mg. gave small but statistically significant ($P < 0.01$) increases in seminal vesicle ratios, but the higher doses of 27 and 81 mg. did not stimulate the tissue. The prostate was somewhat increased at the 3 mg. dose but not at 9, 27 or 81 mg. total dose.

Administration by subcutaneous injection of Ro 2-7239 to the male castrate in parabiosis with an intact female (Table 10) confirmed the stimulation of the accessory organs by this compound, but more striking was the inhibition of the pituitary gonadotropin. This pituitary inhibition can be evaluated by the weight of the ovary. Ro 2-7239 produced a mean ovary weight of 17 ±2 mg. as compared to the vehicle control group of 130 ±17 mg. Ro 2-7239 had a unique effect in preventing ovarian enlarge-

TABLE 10. THE ASSAY OF TESTOSTERONE (T) AND RO 2-7239 (R) BY A PARABIOSIS METHOD BY SUBCUTANEOUS INJECTION

Conditions of Assay: Twenty-four-day-old castrated males and intact females were joined in parabiosis, and the male rat injected subcutaneously once daily for 10 days with 0.5 ml. aqueous suspension of T and R.

Material admin-istered	Total dose, mg.	No. of pairs	Tissue weight, mg. ±S.E.			
			Intact female ovary	Castrated male		
				Seminal vesicle	Prostate	Levator ani
0	0	6	130±17	4.4± 0.37	6.6± 0.92	12±1.9
T	0.4	4	73±24	22 ± 3.4	33 ± 8.0	18±5.0
	0.8	6	26±10	63 ±18	76 ±10	41±7.5
R	100	5	17± 2	12 ± 3	24 ± 5	25±4

ment as compared to the modest androgenicity displayed on the accessory organs. This can be judged in comparison to the action of testosterone studied simultaneously. The magnitude of ovarian inhibition by testosterone at the 0.8 mg. dose level was of about the same order as found for 100 mg. of Ro 2-7239 but testosterone produced seminal vesicles and prostates 8 and 3 times, respectively, larger than the synthetic androgen.

When Ro 2-7239 was administered by gavage at a total dose of 200 mg. over a ten day period to the castrated partner in parabiosis with an intact male, intense pituitary gonadotropin decrease was observed (Table 11). The ovaries of the female parabiont weighed 34 ± 15 mg. as compared to the control ovaries of 104 ± 25 mg. Two hundred mg. of Ro 2-7239 did not cause a significant weight increase in the seminal vesicles, prostate or levator ani muscle.

TABLE 11. THE ASSAY OF METHYLTESTOSTERONE (M) AND RO 2-7239 (R) BY A PARABIOSIS METHOD BY SUBCUTANEOUS INJECTION

Conditions of Assay: Twenty-eight-day old castrated males and intact females were joined in parabiosis and the male treated by gavage once daily for 10 days with 0.5 ml. aqueous suspension of test compound.

Material administered	Total dose, mg.	No. of pairs	Tissue weight, mg. ± S.E.			
			Intact female ovary	Castrated male		
				Seminal vesicle	Prostate	Levator ani
0	0	6	104 ± 25	6.0 ± 0.3	11.8 ± 1.0	15.4 ± 1.1
M	4	5	91 ± 15	10.2 ± 1.1	23.4 ± 1.6	22.5 ± 2.0
	8	5	48 ± 18	21.7 ± 3.1	49.3 ± 6.7	18.2 ± 2.9
R	200	5	34 ± 15	6.6 ± 0.6	14.0 ± 3.7	16.9 ± 3.9

Ro 2-7239 was studied both as a possible estrogen and as an anti-estrogenic substance. The phenanthrene derivative produced no significant increases in uterine weight when injected into groups of 9 to 10 mice at the following dose levels: 1, 10, 100, 200, 400 and 800 μg. By the method employed (6), which involved immature Swiss albino mice, 100% increments in uterine weight were recorded with as little as 0.05 μg. of estrone. In a second estrogen assay where the same method was employed except that the test compounds were administered by gavage, Ro 2-7239 was again inactive at doses up to 100 μg., while estrone was inactive at 0.4 μg.

Ro 2-7239 was tested as an anti-estrogen in the immature female mouse. The mice were stimulated with 0.4 μg. of estrone administered subcutaneously and the test compound injected in one set of experiments and administered by gavage in another. In both tests doses of Ro 2-7239 ranging from one μg. to 4 mg. were inactive.

DISCUSSION

This phenanthrene derivative, Ro 2-7239, is unique in that it is the first nonsteroid found to possess androgenic activity on the basis of the classical criteria. It increases the weight of the chick's comb, simulates the histology seen after testosterone stimulation in the comb, stimulates the weights of the prostate, seminal vesicles and levator ani muscle, and produces histological changes in these tissues comparable to those produced by testosterone.

The fact that the compound can function as an androgen as well as an anti-androgen is analogous to the physiological action of certain estrogens such as estriol. Both compounds produce relatively minimum responses over an extended dose range. Estriol, when administered together with estrone or estradiol-17β, produces uterine hypertrophy and vaginal stimulation lower than that found for the same doses of estrone and estradiol-17β administered alone.

The relative androgenic activity of Ro 2-7239 compared to testosterone is difficult to assess since no log dose-response relation is indicated for the former compound. This synthetic androgen is assigned an activity of 1/100th of testosterone on the chick comb by inunction on the basis of the minimum dose necessary to produce a statistically significant response. On the basis of the castrate rat studies where the compound was injected subcutaneously, a rough estimate of potency indicates a value of 1/400th of testosterone. There is a tendency of Ro 2-7239 to have a relatively greater activity on the levator ani than on the seminal vesicle and prostate glands.

The fact that androgenic activity was demonstrated by direct inunction on the comb argues for an action of the compound *per se*, or a closely related derivative, rather than the compound acting on another gland to produce the effective androgen. In the castrated rat where Ro 2-7239 was injected subcutaneously, the possibility that the adrenal was "stimulated" to produce the androgen requires investigation. In the case of amphenone, for example, it is now known that the progestational activity originally attributed to the compound was due to its action on the adrenal (8). Stimulation of the seminal vesicles, prostate and levator ani of the adrenalectomized-castrated rat by Ro 2-7239 could clarify this point.

The pituitary gonadotropin inhibitory activity of Ro 2-7239 may be as much as ten times as intense as its androgenic activity on the seminal vesicles and prostate. This anti-pituitary activity cannot be explained on the basis of estrogenic activity since the latter type of biological activity could not be demonstrated in the immature female mouse. Further studies are required in this area, particularly to ascertain whether other pituitary tropic hormones are affected.

The activity of Ro 2-7239 by the oral route is reduced as compared to

the activity by subcutaneous administration. More detailed studies are required to establish the relative activities by the two routes. Knowledge of the metabolism of Ro 2-7239 when administered by subcutaneous injection and by gavage and the biological activities of the metabolites may help to elucidate this problem.

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REFERENCES

1. Dorfman, R. I.: *Science*. In Press.
2. Randall, L. O. and J. J. Selitto: *Endocrinology* **62**: 693. 1958.
3. Dorfman, R. I.: *Endocrinology* **64**: 464. 1959.
4. Moore, C. A., D. Price and T. F. Gallagher: *Am. J. Anatomy* **45**: 71. 1930.
5. Moore, C. A., W. Hughes and T. F. Gallagher: *Am. J. Anatomy* **45**: 109. 1930.
6. Rubin, B. L., A. S. Dorfman, L. Black and R. I. Dorfman: *Endocrinology* **49**: 429. 1951.
7. Dorfman, R. I. and A. S. Dorfman: *Acta Endocrinol.* **33**: 308. 1960.
8. Tullner, W. W.: *Proc. Soc. Exptl. Biol. Med.* **98**: 157. 1958.

THE BINDING TO SERUM PROTEIN OF ACETIC AND PROPIONIC ACID ANALOGUES OF THYROXINE AND TRIIODOTHYRONINE[1]

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ABSTRACT

The binding of acetic and propionic acid analogues of thyroxine and triiodothyronine to serum proteins has been investigated by means of a dialysis procedure. Relative to thyroxine, only small quantities of its analogues were found to be in the free, non-protein-bound form. The same was true of the triiodothyronine analogues as compared with the unmodified compound. Addition of the analogues to serum containing labeled thyroxine did not cause an increase in free radiothyroxine indicating that the analogues are bound to other sites than is thyroxine.

In keeping with these results it was found that the uptake of the labeled analogues by erythrocytes was far lower than that of the corresponding iodothyronines.

SINCE the demonstration by Pitt-Rivers (1) of the physiological activity of tetraiodothyroacetic acid (Tetrac) and of triiodothyroacetic acid (Triac), the possibility has been considered that these deaminated analogues are the metabolically active substances. This view was supported by the observation of Thibault and Pitt-Rivers (2) that these analogues had an immediate effect on the oxygen consumption of kidney slices, and also by the findings of Roche *et al.* (3) that Triac could be recovered from rat kidneys following injection of triiodothyronine (T3). The results of Thibault and Pitt-Rivers (2) have, however, not been confirmed (4, 5). Furthermore, since Triac and Tetrac are active only in rather large doses, the hypothesis that the thyroid hormones are active only after deamination has been abandoned.

The fact remains that these analogues are therapeutically active (6, 7, 8). In recent years a number of thorough studies of the biological activities of a large number of substances related to the thyroid hormones have been carried out (9, 10, 11). For many of these substances, the biological activity was found to be largely dependent upon the criterion employed, a phenomenon that has not yet had a satisfactory explanation. For example,

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Triac (6, 12) and triiodothyropropionic acid (13) have proved to possess a marked ability to lower the plasma cholesterol level in doses causing only a slight increase in oxygen consumption. It is well known that several of the phenomena connected with the biological activity of thyroxine (T4) and T3 can be explained in terms of binding of these substances to serum proteins. This might also be true of the acetic and propionic acid analogues of the two hormones. Accordingly, the binding of these substances in the serum has been investigated.

EXPERIMENTAL

Analytical Methods

The dialysis procedure employed has elsewhere been described (14). Briefly the determination is carried out the following way.

A plexiglas vessel consisting of two separate, identical parts is assembled with a cellophane membrane between the two halves. In one of the chambers thus formed is placed 10 ml. of serum, to which radiothyroxine is added (chamber A). In the other chamber (chamber B) is placed 10 ml. of serum. The assembly is incubated in a constant-temperature bath (37.5° C) with adequate stirring of the solution containing the radiothyroxine.

The free I^{131}-T4 will pass through the membrane at a rate proportional to its concentration. During the first 24 hours two samples of 4 ml. are withdrawn from chamber B. At the same time an equal volume is removed from chamber A.

Since the I^{131}-T4 employed contains some inorganic I^{131}, the samples are precipitated by trichloracetic acid and washed carefully, after which the content of I^{131}-T4 is determined in a well-type scintillation counter. The result is expressed as per cent of the total I^{131}-T4 in chamber A.

The uptake by erythrocytes of the I^{131}-labeled compounds was studied in the following manner. One ml. of serum was diluted with 1 ml. phosphate buffer, 0.5 M, pH 7.4, and with 0.9 per cent NaCl to make 10 ml. The radioactive hormone was diluted with physiological saline so that 100 μl. solution contained 0.01 μg. This quantity was added to 2 ml. of the diluted serum, and the mixture was incubated at 37° C for fifteen minutes. Then 2 ml. of a suspension of erythrocytes that had been washed five times with physiological saline was added and the mixture was incubated at 37° C for ninety minutes. It was shaken every fifteen minutes. Afterward, the erythrocytes were washed five or six times with physiological saline, and their content of I^{131} was determined. The amount taken up was expressed in terms of per cent of the total quantity of radioactive material added to the diluted serum after correction of the latter quantity for its content of inorganic I^{131}.

Materials

The I^{131}-labeled thyroxine and the I^{131}-labeled acetic and propionic acid analogues were obtained from the Abbott Laboratories, Chicago. Chromatography showed them to contain 10 to 15% inorganic I^{131}, but no organic impurities. To 10 ml. serum was added 0.1 μg. of the radioactive substance.

RESULTS

The results of the experiments appear in Table 1. It will be seen that the quantities of Tetrac and Tetraprop that pass the membrane in 24

TABLE 1. DIALYSIS EXPERIMENTS AND EXPERIMENTS ON UPTAKE BY ERYTHROCYTES OF LABELED IODOTHYRONINE ANALOGUES

Compound	Per cent passing membrane in 24 hrs.	Per cent taken up by erythrocytes (serum diluted 1:10)
L-Thyroxine	0.50	5.3
Tetrac	0.05	0.6
Tetraprop	0.05	0.85
L-Triiodothyronine	6.00	**
Triac	1.16	8.3
Triprop	0.88	14.0

** The uptake of T3 from the diluted serum was not determined. Since the uptake from the undiluted serum was 8.2%, the theoretical value for the uptake from the serum dilution would be about 10 times this value. For several reasons such excessive uptake is not subject to determination by the erythrocyte procedure.

hours are relatively small, viz., about 10% of the corresponding amounts of T4. Likewise, the quantities of Triac and Triprop passing are far less than that of T3. The results of the experiments on uptake of these substances by erythrocytes are in good agreement with these data. The amounts of Tetrac and Tetraprop taken up by red cells are small relative to that of T4. The uptakes of Triac and Triprop are somewhat higher than that of T4, which is in keeping with the fact that in dialysis experiments they pass the membrane faster than T4.

Table 2 shows the effect on the free radiothyroxine of addition of non-labeled T4, Triac, or Tetraprop to serum. Triac had no effect, and Tetraprop had a slight effect as compared with that observed upon addition of T4.

DISCUSSION

The most reasonable explanation of the experimental results is that only very small quantities of the analogues studied are present in the free, non-protein-bound form. It must naturally be considered whether the molecules of the analogue might not simply pass the membrane with greater difficulty than the parent substance. It would be difficult to check this possibility in a system containing no serum, even in the presence of a carrier. In the first place, a considerable proportion of the substance would be absorbed to the walls of the apparatus and to the membrane, or might be bound to substances extracted from the membrane during the

TABLE 2. DISPLACEMENT OF I^{131}-LABELED THYROXINE FROM ITS BINDING SITES ON PROTEIN BY ADDITION OF 0.25 μG. OF NON-LABELED COMPOUND PER ML. OF SERUM

Non-labeled compound added	Per cent increase in I^{131}-T4 passing membrane
L-Thyroxine	48
Tetraprop	11
Triac	0

experiment. In the second place, the solubilities of the analogue might make themselves felt. In the third place, instability phenomena of the type described by Tata (15) might make the interpretation of such experiments difficult.

The experiments on uptake by erythrocytes support the view that the dialysis results must be explained by the assumption that the analogues are very firmly protein-bound. The fact that the uptake by red cells of, for example, Tetrac, is very slight as compared with that of T4 might be due to a far lower affinity to the binding sites in the red cells of Tetrac than of T4. The only probable explanation which satisfies both the dialysis and the red cell uptake results is, however, that only small quantities of the analogues are present in the free form. This constitutes strong evidence that it is correct.

If it is assumed, as a first approximation, that the law of mass action applies to the association of the iodothyronine analogues with the serum proteins, the following equation will hold:

$$[A_f] = K \frac{[AP]}{[P_{unsat}]}$$

where $[A_f]$ and $[AP]$ denote the concentration of the free analogue and the analogue-protein complex respectively, and $[P_{unsat}]$ that of unsaturated receptor sites on the proteins. According to this, the low value of $[A_f]$ found can be explained either by a high binding affinity (as expressed by $1/K$) between the protein and the analogue, or by a very high concentration of unsaturated binding sites. Paper electrophoretic experiments by Tanaka and Starr (16) have shown that the thyroid hormone analogues studied in this work are bound almost exclusively to the albumin and prealbumin. They found that the receptors on these proteins could not be saturated with the analogues even when the latter were added to serum to concentrations exceeding 100 μg. per 100 ml. Ingbar (17) has shown that the analogues are preferentially bound to prealbumin. These results are in accord with the findings of Robbins and Rall (18) that the thyroxine-binding globulin binds only very small quantities of the analogues. The experiments listed in Table 2 are also in keeping with these observations. The fact that addition of the non-labeled analogues does not result in any appreciable increase in free T4 suggests that they are chiefly bound to other sites than is T4 as far as sera with a normal content of T4 are concerned.

Thus, the low concentrations of the free forms of the analogues are best explained by the presence in serum of a high concentration of receptor sites.

It is well known that T3 is metabolized much more rapidly in the organism than is T4, and part of the explanation offered for this is that T3 is

far more loosely bound to the serum proteins. Accordingly, it might be anticipated that the analogues studied would be metabolized more slowly than the parent substances and that it would be difficult for them to enter the body cells. To the author's knowledge, no investigation on the disappearance rates of these substances from human plasma has been reported.

Larson and Albright (19) have investigated the metabolism of Triac injected into rats. In contrast to T3, it is not taken up to an appreciable extent by the kidneys or by skeletal muscle, which is in accord with the view that most of it is protein-bound. On the other hand, Triac leaves the plasma at approximately the same rate as T3. It is preferentially excreted with the bile, and possibly it is not easily reabsorbed. No studies of the mechanism by which these analogues pass the intestinal wall are available. Wilkinson (20) followed the deiodination *in vivo* of Triac and Tetrac in rats by determining the excretion of inorganic iodine in the urine. It appeared that the two compounds were deiodinated much less rapidly than T3 and T4. The overall rate of excretion of Tetrac in urine and feces was much lower than those of the other compounds. These results may be interpreted in terms of binding to protein and are in accord with the findings presented here. In experiments *in vitro*, Maclagan and Reid (21) found that Triac and Tetrac were deiodinated slowly by a liver extract. Insofar as the deiodination rates may possibly depend on the concentrations of the free, non-protein-bound compounds, these results are also in accord with the views advanced in the present communication.

However, no studies on the binding of the analogues to rat serum proteins have been performed, and considerable species differences in binding properties may well be found.

One might speculate whether part of the metabolic effect of the analogues is due to a blocking of some of the thyroxine-binding sites, resulting in an increase in free T4. The data of Table 2 speak against this hypothesis.

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REFERENCES

1. Pitt-Rivers, R.: *Lancet* **2**: 234. 1953.
2. Thibault, O. and R. Pitt-Rivers: *Lancet* **1**: 285. 1955.
3. Roche, J., R. Michel, P. Jouan and W. Wolf: *Endocrinology* **59**: 425. 1956.
4. Barker, S. B.: *Endocrinology* **59**: 548. 1956.
5. Wiswell, J. G. and S. P. Asper: *Bull. Johns Hopkins Hosp.* **102**: 115. 1958.
6. Lerman, J. and R. Pitt-Rivers: *J. Clin. Endocrinol. and Metab.* **15**: 653. 1955.
7. Trotter, W. R.: *Lancet* **2**: 374. 1955.
8. Goolden, A. W. G.: *Lancet* **1**: 890. 1956.
9. Selenkow, H. A., C. A. Plamendon, J. G. Wiswell and S. P. Asper: *Bull. Johns Hopkins Hosp.* **102**: 94. 1958.

10. Stasilli, N. R., R. L. Kroc and R. I. Meltzer: *Endocrinology* **64:** 62. 1959.
11. Rawson, R. W., W. L. Money, R. L. Kroc, S. Kumaocha, R. S. Benua and R. D. Leeper: *Trans. Assoc. Am. Phys.* **72:** 225. 1959.
12. Trotter, W. R.: *Lancet* **1:** 885. 1956.
13. Bauer, H. G., T. H. McGavack and L. Swell: *J. Clin. Endocrinol. and Metab.* 19: 490. 1959.
14. Christensen, L. K.: *Scandinavian J. Clin. Lab. Invest.* **11:** 326. 1959.
15. Tata, J.: *Biochem. J.* **72:** 214. 1959.
16. Tanaka, S. and P. Starr: *Acta Endocrinol.* **31:** 161. 1959.
17. Ingbar, S. H.: *Ann. N. Y. Acad. Science* **86:** 440. 1960.
18. Robbins, J. and J. E. Rall: *J. Clin. Invest.* **34:** 1331. 1955.
19. Larson, F. C. and E. C. Albright: *Endocrinology* **63:** 183. 1958.
20. Wilkinson, J. H.: *Biochem. J.* **73:** 334. 1959.
21. Maclagan, N. F. and D. Reid: Ciba Found. Colloquia on Endocrinology **10:** 190. 1957

PURIFICATION OF THYROID PROTEASES BY CELLULOSE COLUMN CHROMATOGRAPHY

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ABSTRACT

Several methods for purification of thyroid protease have been investigated. Chromatography on diethylaminoethyl and carboxymethyl cellulose resulted in purification and separation of two distinct acidic proteases. One protease showed a pH optimum of 3.8 and the other of 5.7. A combination of chromatography on both types of cellulose colums resulted in 500 fold purification of both enzymes.

SEVERAL proteolytic enzymes have been found in thyroid extracts (1, 2, 3). The best studied enzyme showed optimal activity around pH 3.5 (2). The present paper describes a procedure for the chromatographic separation of two proteolytic enzymes from sheep thyroid extract with approximately 500 fold purification.

MATERIALS AND METHODS

Thyroid glands were collected in the abattoir from freshly killed sheep, and were carried on ice to the laboratory where they were cleaned of adventitious tissue and homogenized in a Waring blendor with chilled distilled water. The homogenate, which had a concentration of 0.25 gm. wet weight of thyroid per ml., was frozen over night to facilitate extraction. The extract was thawed and filtered through a fine nylon mesh.

For assaying the proteolytic activity, a modification of Anson's hemoglobin method was used (4). A 2% bovine hemoglobulin solution, buffered to the required pH, was used as substrate. Five ml. of the 2% hemoglobulin solution was incubated with 0.5 ml. of thyroid extract, or of other enzyme solution, in a constant temperature water bath at 31° C for 10 minutes. The reaction was terminated by the addition of 5 ml. of 10% trichloracetic acid solution. Following centrifugation, the supernatant fluid was diluted with distilled water and the ultraviolet absorption recorded at 280 mμ in a Beckman model DU spectrophotometer. The activity was expressed in terms of per cent of a standard 1:1,000 solution of tyrosine per 0.5 ml. or per 1 mg. of nitrogen of the enzyme solution used.

Extracts containing I^{131}-labeled thyroglobulin were prepared by incubating sheep thyroid slices with radioactive iodide-I^{131} in Krebs-Ringer bicarbonate buffer pH 7.4 in a constant temperature water bath at 37° C for 2 hours with constant shaking and in an atmosphere of 95% oxygen and 5% CO_2. The slices were then extracted as described above. The extract was dialyzed against distilled water.

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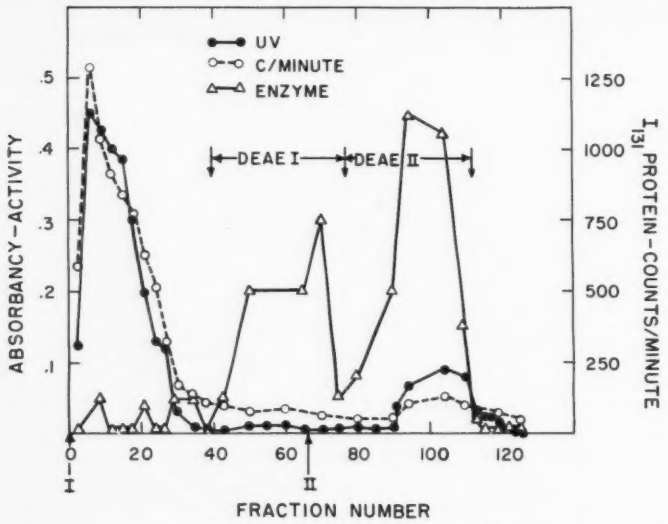

FIG. 1. Chromatogram of 80 ml. sheep thyroid extract on DEAE cellulose 2.2×30 cm. column. Effluent collected in 3 ml. fractions. Temperature, 2° C. Solvents: I, 0.01 M sodium phosphate, pH 6.8, beginning with distilled water in the mixing chamber; II, 0.1 M sodium phosphate, pH 6.8. Mixing chamber volume, 200 ml.; •, ultraviolet absorption at 280 mμ; ○, counts/minute/1 ml. of effluent labeled with I^{131}; △, protease activity in % of tyrosine 1/1,000 standard solution per 0.5 ml. of effluent.

Protein concentration in the enzyme solution was estimated by two procedures: (1) The absorption at 280 mμ was measured for diluted aliquots of the enzyme solution. The thyroid extracts studied were found to have absorption values corresponding to an extinction coefficient of 7.3 for a 1% solution. (2) The total nitrogen content was measured by a modified micro-Kjeldahl method (5).[2]

Diethylaminoethyl-cellulose (DEAE) was purchased from Eastman Kodak Chemicals, Rochester 3, N. Y. It was washed in acid and base and then with distilled water before use.

Carboxymethylcellulose (CMC) was a gift of Dr. Peter G. Condliffe. This was washed with acid and base and then was equilibrated with 0.001 M phosphate buffer (equimolar K_2HPO_4 and KH_2PO_4) at pH 6.0.

Bentonite, U.S.P. Powder, was purchased from Fischer Scientific Company, Fair Lawn, N. J. Hyflo Super-Cel was purchased from Johns-Manville Products, Baltimore, Md. Both powders were used separately and in a mixture.

Fractionation Methods

(1) *Column Chromatography on DEAE (Diethylaminoethyl)-cellulose.* In an illustrative experiment, 80 ml. of thyroid extract were placed on a 2.2×30 cm. DEAE-cellulose column. Elution was carried out with a gradient of phosphate buffer (equimolar K_2HPO_4 and KH_2PO_4) beginning with

[2] We are indebted to Dr. William C. Alford for these determinations.

distilled water and approaching a concentration first of 0.01 M and then of 0.1 M (pH 6.8) using a constant volume mixing device (200 ml.) similar to that described by Alm *et al.* (6). Three ml. effluent fractions were collected. Eighty per cent of the protein in the extract was recovered in the initial 40 fractions. A similar proportion of radioactive I^{131}-labeled protein, used as an indicator, was recovered in this initial phase of elution (Fig. 1). Over 90% of the enzyme activity was recovered in the following fractions (Fig. 1): (1) DEAE I, which was found in fractions 43 through 71 and (2) DEAE II, which was recovered in fractions 88 through 112.

(2) *Column Chromatography on CMC (Carboxymethylcellulose).* The 2 fractions recovered from the DEAE column of 80 ml. thyroid extract, DEAE I & II, were mixed and dialyzed against distilled water. They were then placed on a 2.2×30 cm. column containing about 10 gm. of carboxymethyl cellulose. A gradient of phosphate buffer eluent was applied beginning with 0.001 M phosphate and approaching 1.0 M (pH 6.8) in three gradients (Fig. 2). The mixing chamber contained initially 200 ml. of 0.001 M phosphate buffer. Three ml. effluent fractions were collected. About 30% of the protein initially present in the DEAE fractions was

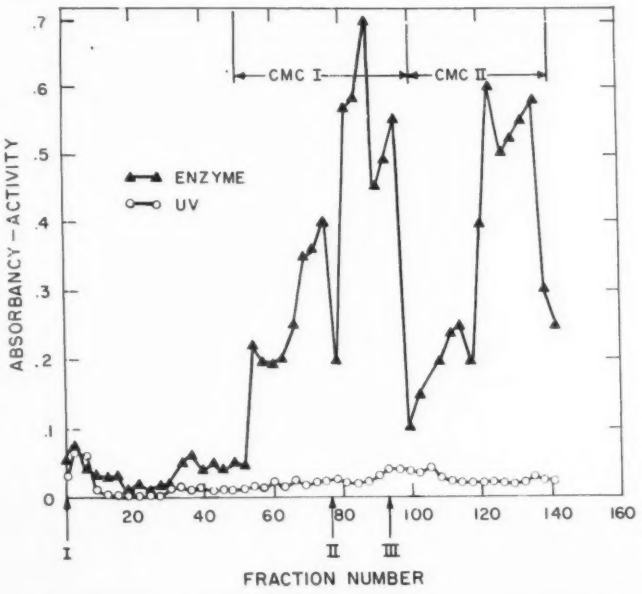

FIG. 2. Chromatogram of DEAE fractions I and II recovered from 80 ml. thyroid extract on CMC 2.2×30 cm. column. Effluent collected in 3 ml. fractions. Temperature, 2° C. Solvents: I, 0.01 M sodium phosphate, pH 6.8, beginning with 200 ml. 0.001 M sodium phosphate, pH 6.8, in the mixing chamber; II, 0.1 M sodium phosphate, pH 6.8; III, 1 M sodium phosphate, pH 6.8; ○, ultraviolet absorption at 280 mμ; ▲, protease activity in % of tyrosine 1/1,000 standard solution per 0.5 ml. of effluent.

recovered in the initial 50 CMC fractions. About 80% of the enzyme activity put on the column was recovered in the following fractions (Fig 2): (1) CMC I, which was obtained in fractions 51 through 100 and (2) CMC II, which was obtained in fractions 101 through 141.

The enxyme fractions from the DEAE and CMC columns were studied in the analytical ultracentrifuge (Spinco model E).[3] The DEAE fractions showed a rapidly sedimenting peak, presumably thyroglobulin. This band was eliminated in the product obtained after adsorption and elution from both columns.

(3) *Physical Adsorption.* The protease enzyme and the proteins in sheep thyroid extract could be adsorbed on various materials, such as: 1. Bentonite; 2. Hyflo Super-Cel; 3. calcium phosphate gel; 4. aluminum C gamma gel. Calcium phosphate gel and aluminum C gamma gel were abandoned because the enzyme could not be successfully eluted from the gel. The enzymes however could be adsorbed and partially eluted from a mixture of bentonite and Hyflo Super-Cel. With increasing molarity of NaCl, only about 10–15% of the enzyme could be recovered from either adsorbent with a 50 fold increase in specific activity.

(4) *Thyroglobulin Fractionation.* Ultracentrifugation (7) and salting out with phosphate buffer or ammonium sulphate (8) were used to fractionate thyroglobulin. Using I^{131}-labeled thyroglobulin as an indicator, ultracentrifugation (two spins at 144,000 × g for $3\frac{1}{2}$ hours) sedimented about 80% of the proteins, whereas over 40% of the protease was recovered in the supernatant fluids. With salting out, significant separation of the protease from thyroglobulin was observed at a phosphate concentration of 2.45 M (equimolar NaH_2PO_4 and Na_2HPO_4) or when a concentration of 45% saturated ammonium sulphate was reached.

RESULTS AND DISCUSSION

In Table 1, the recovery and specific activity of the fractions obtained from the DEAE & CMC columns are summarized for a typical experiment on 80 ml. of sheep thyroid extract. It may be noted that the chromatographic data demonstrate two elution peaks. The enzyme was active between pH 2.8 and 6.0. However, two peaks were present: one at pH 3.8 and the other at 5.7 (Fig. 3).

When the proteolytic activity in the fractions obtained from the DEAE & CMC columns were examined as a function of the pH, it was found that DEAE I and CMC I had a pH optimum at 3.6 and DEAE II and CMC II at 5.7 (Fig. 3). Consequently the recovery and specific activity were studied at these pH's.

It is apparent from these data that two distinct proteases, with different pH optima, are present in the thyroid. These can be satisfactorily sepa-

[3] We are indebted to Dr. Harold Edelhoch for the ultracentrifuge studies.

TABLE 1. SPECIFIC ACTIVITY (IN TERMS OF % TYROSINE STANDARD 1:1000/MGM. NITROGEN) AND RECOVERY (IN TERMS OF PERCENTAGE OF EXTRACT'S TOTAL ACTIVITY) OF PROTEASE ENZYME RECOVERED IN SHEEP THYROID EXTRACT AND ITS FRACTIONS FROM CELLULOSE COLUMN CHROMATOGRAPHY (DEAE & CMC)

	Fraction	Specific activity	Recovery
pH 3.6	Extract	.23	100
	DEAE I (43–71)	4.0	84
	CMC I (51–100)	120.0	80
pH 5.7	Extract	.14	100
	DEAE II (88–112)	5.3	86
	CMC II (101–141)	92.3	69

rated by chromatography on either DEAE or CMC. When both exchangers were used in sequence, an approximately 500 fold purification of each protease was achieved.

Further studies are necessary to determine the specificity of each of the two thyroid proteases obtained. The physiological significance of the two enzymes is even more difficult to ascertain. It is evident that some protease is necessary for the hydrolysis of thyroglobulin *in vivo* since almost all the thyroxine in the thyroid is present in that protein. It is still not clear whether either of the proteases obtained in this study are responsible

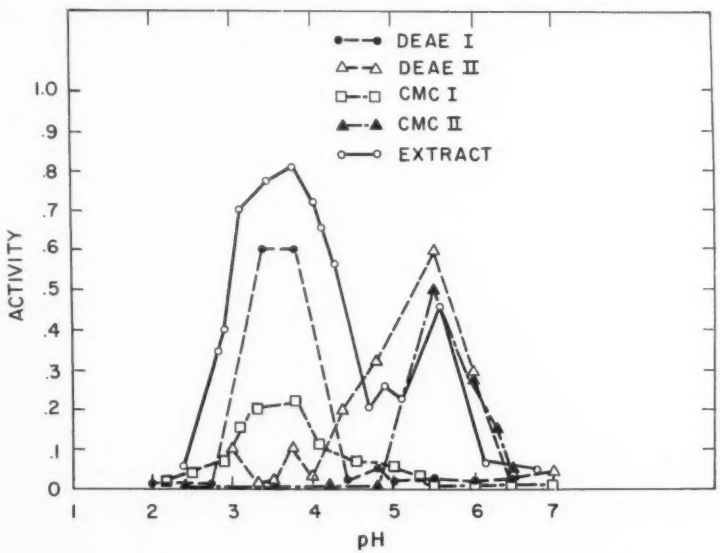

FIG. 3. Thyroid protease activity (expressed in terms of % of tyrosine 1/1,000 solution per 0.5 ml. of enzyme solution) in thyroid extract and its DEAE & CMC purified fractions at pH range 2–7).

for the physiological hydrolysis of thyroglobulin. The previous finding of DeRobertis (1) and Watson and Trikojus (9) that thyrotropic hormone increases the amount of protease in the thyroid implies that at least one of these proteolytic enzymes may have a physiologic role.

REFERENCES

1. DeRobertis, E.; *Anat. Record* **80:** 219. 1941.
2. McQuillan, M. T. and V. M. Trikojus: *Australian J. Biol. Sci.* **6:** 617. 1953.
3. Alpers, J. B., M. L. Petermann and J. E. Rall: *Arch. Biochem. Biophys.* **65:** 513. 1956.
4. Anson, M. L.: *J. Gen. Physiol.* **22:** 79. 1938.
5. Ma, T. S. and G. Zuazaga: *Ind. Eng. Chem., Anal. Ed.* **14:** 280. 1942.
6. Alm, R. S., R. J. P. Williams and A. Tiselius: *Acta Chem. Scand.* **6:** 826. 1952.
7. Robbins, J., M. L. Petermann and J. E. Rall: *J. Biol. Chem.* **208:** 387. 1954.
8. Robbins, J.: *J. Biol. Chem.* **208:** 377 1954.
9. Watson, P. and V. M. Trikojus: *Ann. Chem. Liebigs* **607:** 215 1957.

CONJUGATES OF TRIIODOTHYRONINE AND ITS METABOLITES[1]

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ABSTRACT

Compounds *a* and *b*, previously described as major metabolites formed in the dog from 3,5,3′-I^{131} triiodothyronine (T3), have been identified by enzymatic and chromatographic technics as T3 sulfate and 3,3′ T2 sulfate. These sulfoconjugates occur together in the bile in amounts equal to or greater than that of T3 glucuronide; and with a third, but unidentified, sulfate they account for 90% of the I^{131} excreted in the urine by the dehepatized dog after the injection of radioactive T3. The major metabolite of T3 in blood and urine of the dehepatized dog is 3,3′ T2 sulfate. Deiodination of T3 by extrahepatic tissues is extensive, but it involves chiefly the removal of iodine (nonradioactive in the compound given) from the benzene ring with the alanine side chain, and to a very limited extent, the I^{131} from the phenolic ring.

CONJUGATION with glucuronic acid constitutes an important metabolic pathway for thyroxine (T4) (1, 2) and triiodothyronine (T3) (3, 4). The glucosiduronic acids of these substances normally are excreted in the bile, and small amounts appear in the urine. Much larger amounts are excreted in the urine when the bile duct is obstructed. Glucuronides of both thyroxine and a derivative are excreted in large amounts in the urine of dehepatized dogs also (5), and are present even in the blood of dehepatized-nephrectomized dogs. Curiously, only small amounts of T3 glucuronide are excreted in the urine of dehepatized dogs injected with T3, and the major products in the urine are two acid-labile compounds, *a* and *b*, not hitherto identified (5). Normally these compounds are excreted chiefly in the bile, but they appear also in the urine of dogs with biliary fistulas and in larger amounts in the urine of dogs with biliary obstruction (2, 4). Gross and co-workers (6) have found an acid-labile compound of T3 in the bile, plasma, liver, and kidney of the rabbit.

Conjugation of T3 with sulfuric acid has been demonstrated in the rat (7–9) and in man (10) following administration of I^{131}-labeled T3 and S^{35}-labeled sodium sulfate. The conjugate has been shown to be identical

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with synthetic T3 sulfate (8, 9). This sulfoconjugate is excreted in the bile and also circulates in the plasma.

We have used mylase P, which contains an arylsulfatase (11), to study the acid-labile I^{131} compounds that appear in bile and urine of dogs with biliary fistulas, and also in urine and plasma (12) of liverless dogs following administration of triiodothyronine.

METHODS

Dogs weighing from 5.9 to 19.7 Kg. were used in the study. Intravenous injections of I^{131}-labeled T3 or T4 were given to some of them 1 or 2 hours after total hepatectomy (13), and to other dogs 1 hour to 5 days after cannulation of the bile duct. The doses of these radioactive hormones per Kg. of body weight varied from 8 to 84 μc. and from 0.5 to 1.9 μg. To the dehepatized dogs saline, or a mixture of saline and dextran, was administered by continuous intravenous injection at the rate of 20 ml. per hour; and glucose, dissolved in the saline, was given at the rate of 150 mg. per Kg. per hour for 4 to 12 hours and then at higher rates up to 350 mg. per Kg. per hour when indicated.

Urine and bile were collected continuously throughout the experiment, and blood was collected at intervals. The radioactivity of the urine, bile, and plasma was measured in a well-type NaI (thallium-activated) scintillation counter. The amount of I^{131} circulating in the plasma was calculated on the assumption that 4.5% of the body weight of the dog was plasma. The compounds of I^{131} were separated by column and paper chromatography, and then incubated with β-glucuronidase, mylase P, or acid.

Preparation of Extracts.—Urine, bile, and plasma were extracted in most of these experiments with four volumes of methanol-acetone (1:1), and centrifuged. The precipitate was washed once with a few milliliters of methanol-acetone. The extract was dried by standing in an open beaker at room temperature, or with reduced pressure at 30° C. By this procedure more than 90% of the I^{131} is recovered in a protein-free residue of small bulk, which is suitable for chromatography.

Column Chromatography.—The dried extract from a few milliliters of bile or from as much as 10 or 20 ml. of plasma or urine was dissolved first in a small amount of the stationary phase and then in the mobile phase (4:1 butanol-chloroform-0.5N NaOH), and pipetted onto the kieselguhr column. The I^{131} compounds were eluted first with 4:1 butanol-chloroform saturated with 0.5N NaOH, then with 9:1 butanol-propanol, also saturated with 0.5N NaOH, and finally with water as previously described (4, 5). The radioactivity of the effluent was recorded continuously, and the effluent was collected in test tubes (7.5 ml. per tube) by a fraction collector. After the radioactivity in the collecting tubes was measured in the well-type scintillation counter, the total radioactivity in each peak was calculated. The effluent in the column peaks was dried under reduced pressure.

Paper Chromatography.—The dried methanol-acetone extracts were dissolved in a small volume of 50% ethanol. Aliquots were used for two-dimensional chromatography on 10 by 12 inch sheets of Whatman 3-MM filter paper, and for one-dimensional chromatography on 1.5 by 22 inch strips of the same paper. The strips were developed for 20 to 24 hours in butanol-dioxane-2N ammonia (BDA). The sheets were developed in the same solvent overnight, then in tertiary amyl alcohol ammonia (TAA) for 2 days. Sometimes the TAA solvent was replaced by another solvent: namely, butanol-acetic acid-water (78:5:17) (14) or methanol-0.2M ammonium acetate (1:2.5) (15). Radioautographs were made from the sheets on No-Screen x-ray film and from the strips on Eastman Linagraph Ortho film. Radioactivity on the strips was often measured and recorded by the Forro Chromatogram Scanner. Nonradioactive markers of T3, ri-

iodothyroacetic acid (triac), 3,3′ diiodothyronine (3,3′ T2), tetraiodothyroacetic acid (tetrac), and T4 were located by use of diazotized sulfanilic acid, and iodide by use of palladium iodide.

Hydrolysis of I^{131} Compound.—I^{131} compounds were incubated with β-glucuronidase at 37° for 60 minutes, then extracted with butanol; and the hydrolytic products were chromatographed on paper. I^{131} compounds also were incubated with mylase P at pH 6.5 at 50° overnight (16, 17), then extracted with butanol and chromatographed. Hydrolysis of compound *b* with mylase P was found to be 28% complete in 1 hour, 54% in 3 hours, 81% in 6 hours, and 86% in 24 hours. Also, I^{131} compounds were incubated with 2N HCl at 50° for 1 hour, then extracted and chromatographed.

TABLE 1. DISTRIBUTION OF I^{131} IN DOGS INJECTED WITH T3

Dog No.	Hours after injection	I^{131} content, per cent of dose		
		Plasma	Urine	Bile
		Hepatectomy		
1	6	16.4	2.7	
2	12	17.3	20.7	
3	12	9.1	1.1	
4	16	10.2	0.7	
5	21	15.5	1.8	
6	22	8.0	28.0	
7	24	—	18.0	
8	24	6.2	19.9	
		Biliary fistula		
9	6	—	2.4	15.4
10	6	—	5.2	15.6
11	6	6.4	25.1	31.5
12	18	—	16.3	7.3
13	24	2.4	57.6	11.0
14	24	2.1	49.3	14.4
15	24	7.4	12.8	7.8
15*	24	—	17.0	5.5
16	27	1.6	42.6	5.4

* A second injection of T3 was given to this dog 6 days after the first injection.

RESULTS

Dehepatized dogs in good condition excreted in urine approximately one fifth of the dose of I^{131} after intravenous injection of I^{131}-labeled T3 (Table 1). Most of this I^{131} was found in compounds which were hydrolyzable with mylase P and thus presumably were sulfates. Two of these compounds, *a* and *b*, came off the kieselguhr column before inorganic iodide and overlapped the T3 region; a third compound, *d*, came off the column after the iodide (Fig. 1). These three compounds accounted for most of the I^{131} in the urine. In plasma, most of the I^{131} was accounted for in compounds *a*, *b*, and *c*, which came off before iodide.

Compounds *a*, *b*, and *d* clustered close to, but at the right of, iodide on two-dimensional paper chromatograms (Fig. 2). Compounds *a* and *b* often were unresolved, particularly on the two-dimensional chromatograms; but

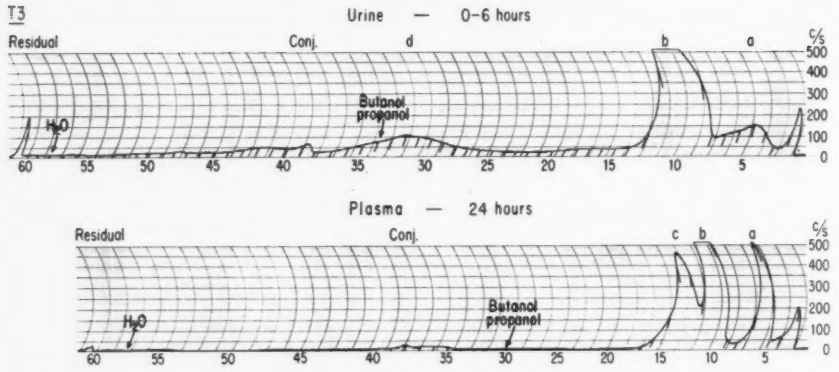


FIG. 1. *Above*, column chromatogram of urine from a dehepatized dog after injection of T3. Total I^{131} excreted in 6 hours was 4.8% of dose. Compound *a* (T3 sulfate) was 9% of the urinary I^{131}; compound *b* (3,3′ T2 sulfate) was 61%; and compound *d* (X2 sulfate) was 14%. *Conj.* is T3 glucuronide.

Below, column chromatogram of plasma. Total I^{131} in plasma was 15.5% of dose. Compound *a* (T3 sulfate) was 20% of the plasma I^{131}; compound *b* (3,3′ T2 sulfate) was 46%; and compound *c* (an unidentified sulfate) was 25%. *Conj.* is T3 glucuronide.

compound *d* usually was clearly separated from these and T3 glucuronide (Figs. 2 and 3).

T3 was liberated from compound *a* when it was incubated with mylase P (Fig. 4*a*). Hence it appears that our compound *a* is identical with the T3 sulfate that Roche and co-workers have identified in the bile of the rat after the administration of T3 (8, 9). The product liberated from compound *b* by mylase P, when run on two-dimensional paper chromatograms in butanol-dioxane-ammonia and tertiary amyl alcohol ammonia, usually overlapped the lower part of the tetrac marker or when present in large amounts covered it (Fig. 4*b*). We found that this product coincides exactly with the 3,3′ T2 marker (Fig. 5). It also coincides with the 3,3′ T2 when two other solvent combinations are used, namely BDA and butanol-acetic acid-water (78:5:17) and BDA with methanol-0.2M ammonium acetate (1:2.5). Thus it now appears that our compound *b* is 3,3′ T2 sulfate. When compound *d* was incubated with mylase P, an unidentified product, X2, was liberated (Fig. 4*c*). It had lower Rf values than 3,3′ T2 on paper chromatograms and usually overlapped the lower portion of the T4 marker. The unidentified hydrolytic product from compound *c* in plasma traveled just beyond iodide in the butanol-dioxane-ammonia system.

Acid hydrolysis of urinary compounds *a*, *b*, and *d* in 2N HCl at 50° for 1 hour liberated the same products as did mylase P (Fig. 6).

In the liverless dog, sulfoconjugates of T3 and 3,3′ T2 and an unidentified metabolite, X2, accounted for about 80% of the I^{131} excreted in the urine, while glucuronoconjugates accounted for less than 5%. Also in the

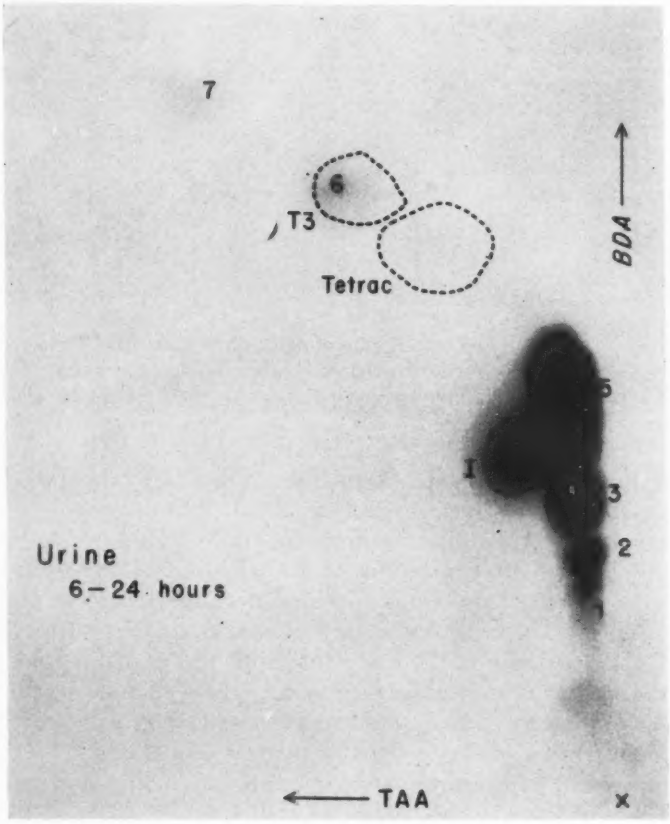


FIG. 2. Paper chromatogram of urine collected from dog represented in Figure 1 during period 6 to 24 hours after injection of T3. Total I^{131} excreted in sample was 15.1% of dose. Spot 2 (T3 glucuronide) contained 4% of urinary I^{131}. Spot 3 (compound *d*, or X2 sulfate) contained 15%. Spot 4 (iodide) contained 4%. Spot 5 (*a* and *b* combined, or T3 sulfate and 3,3′ T2 sulfate) contained 73%. Positions of markers are indicated by dashed lines.

plasma of the dehepatized dog, most of the I^{131} was present as the sulfoconjugates T3 sulfate, 3,3′ T2 sulfate (12), and an unidentified compound *c*. The most abundant sulfoconjugate in both urine and plasma was 3,3′ T2 sulfate.

After the administration of I^{131}-labeled T3 to normal dogs, the sulfates of T3 and of its metabolite 3,3′ T2 were excreted in the bile in amounts equal to or even greater than that of T3 glucuronide (Fig. 7). The amount of I^{131} excreted in the bile in 3,3′ T2 sulfate was much greater than that excreted in T3 sulfate. Both 3,3′ T2 sulfate and T3 sulfate were excreted in the urine of the dogs with biliary fistulas, but the quantity was much

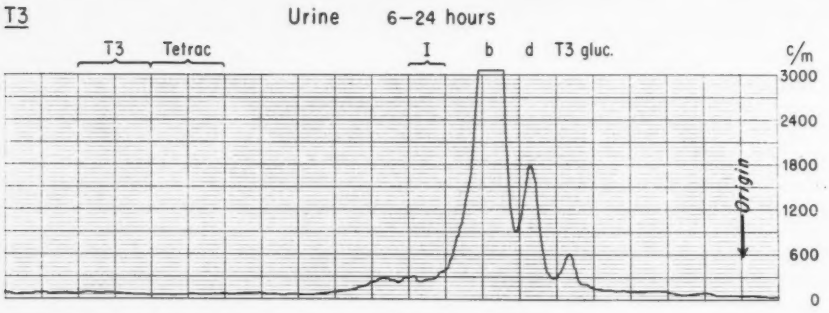


FIG. 3. Same urine as in Figure 2. Record of radioactivity on a one-dimensional paper chromatogram made by Forro chromatogram scanner. In this separation, compound *a* is included with compound *b* under heading b. Positions of markers on strip are indicated by brackets.

less than that of inorganic iodide. In this study 3,3′ T2 was not detected in the free state, except after hydrolysis of 3,3′ T2 sulfate.

Also, mylase-hydrolyzable I^{131} compounds have been found in the urine of dehepatized dogs after injections of T4. These came off the kieselguhr column ahead of the iodide and appeared near the iodide on the paper chromatograms. In a urine specimen collected from the 6th to the 23rd hour after the injection of T4 and containing 10.2% of the dose of I^{131}, three sulfoconjugate compounds were found. These sulfates accounted for 48% of the I^{131} in the urine, and two glucuronides accounted for 44%. After administration of T4 to dogs with biliary fistula, a mylase-hydrolyzable compound has been found in the bile; but the amount was much less than that of the T4 glucuronide.

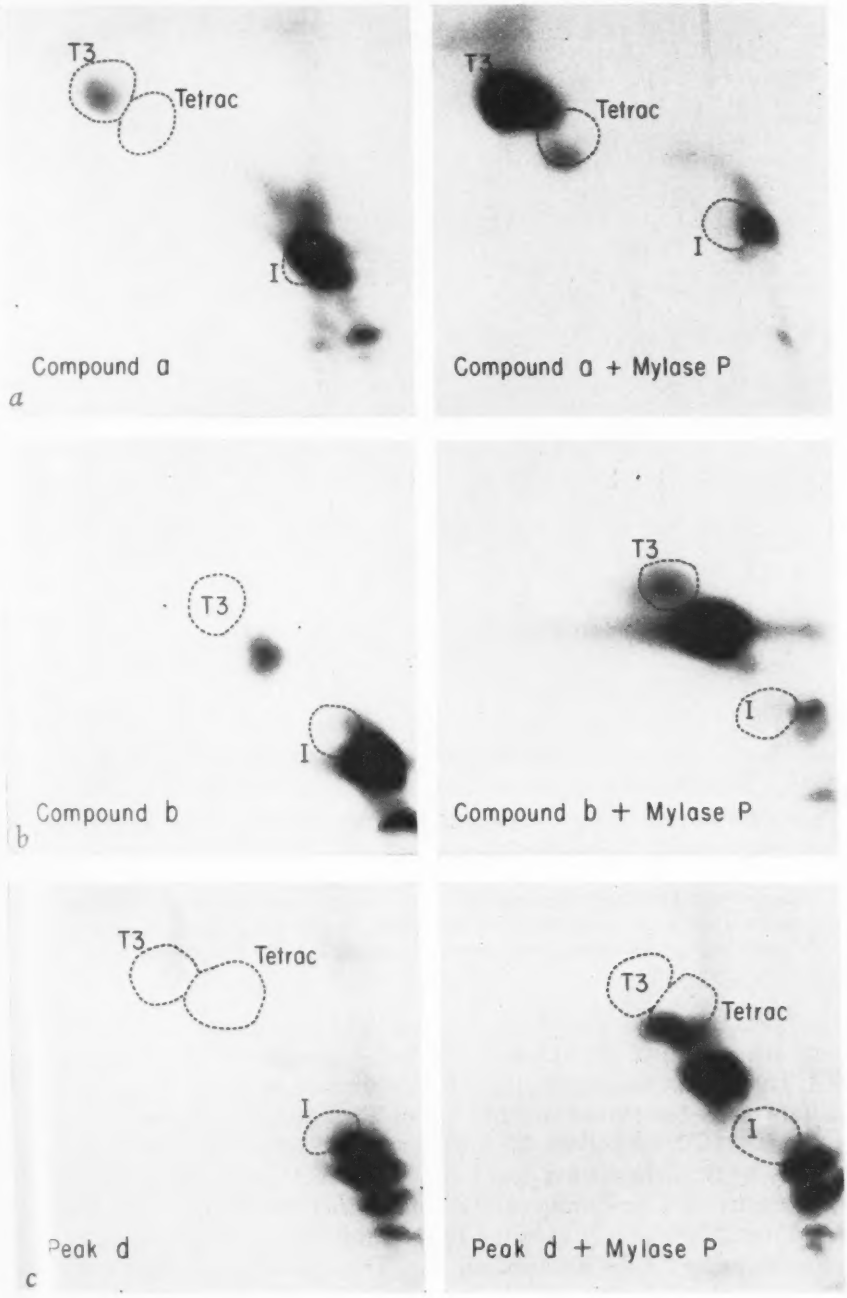
COMMENT

The euthyroid dog, like the thyroidectomized rat studied by Roche and co-workers (8, 9), conjugates T3 with sulfate and excretes T3 sulfate in

FIG. 4. *a*. Paper chromatograms of compound *a* (T3 sulfate from the column shown in Fig. 1) after incubation with and without mylase P. The major hydrolytic product was T3. Markers are indicated by dashed lines. The chromatograms have been trimmed to a 7-inch square containing the radioactivity.

b. Paper chromatograms of compound *b* (3,3′ T2 sulfate in urine from strip shown in Fig. 3) after incubation with and without mylase P. The major hydrolytic product was 3,3′ T2 (see also Fig. 5).

c. Paper chromatograms of compound *d* (X2 sulfate in urine from column shown in Fig. 1) after incubation with and without mylase P. The major hydrolytic product was X2, which appears below the Tetrac marker.


T3
Tetrac
I
Compound a
a
T3
Tetrac
I
Compound a + Mylase P
T3
I
Compound b
b
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I
Compound b + Mylase P
T3
Tetrac
I
Peak d
c
T3
Tetrac
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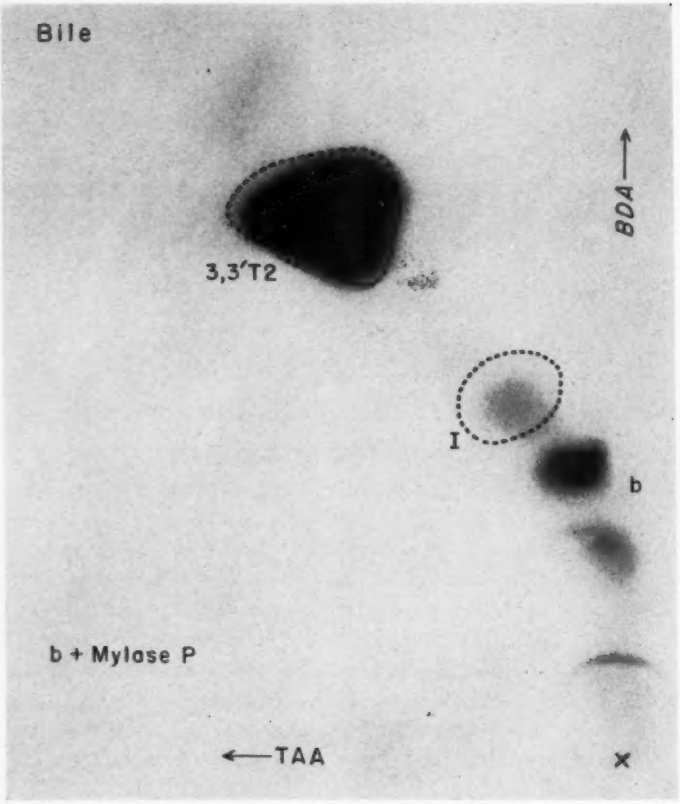

FIG. 5. Paper chromatogram of compound *b* (3,3′ T2 sulfate) from bile after incubation with mylase P. The hydrolytic product coincided with the 3,3′ T2 marker. A small amount of compound *b* remained unhydrolyzed.

Compound *b* had been separated first on a column chromatogram and then on one-dimensional paper chromatograms before incubation with the enzyme. Without enzyme treatment most of the radioactivity was at b and no 3,3′ T2 was present.

the bile. After injection of T3, however, the dog excretes in the bile not only the sulfate of T3, but also even larger amounts of the sulfate of 3,3′ T2. The liver is not essential for the conjugation of the hormone with the sulfate or for the partial deiodination of T3 that occurs during the formation of 3,3′ T2, since both T3 sulfate and 3,3′ T2 sulfate are synthesized readily by the dehepatized dog. Indeed, the chief metabolite of T3 in both the plasma (12) and urine of the dehepatized dog is 3,3′ T2 sulfate. A third metabolite also is excreted in the urine in significant amounts as a sulfoconjugate by the dehepatized dog. The hydrolytic product from this conjugate showed progressively lower Rf's than T3 and 3,3′ T2 on paper

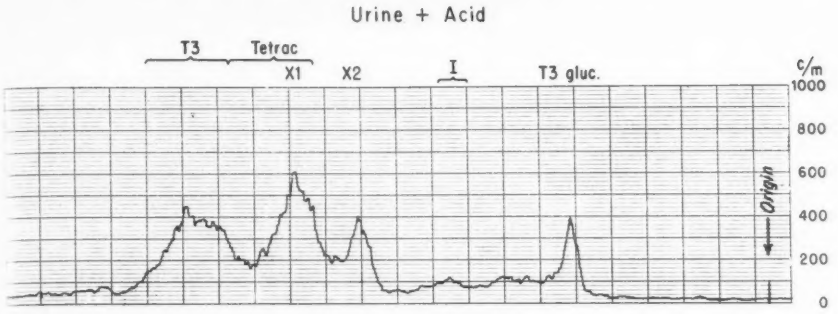

FIG. 6. Effect of acid hydrolysis of urine excreted by a dehepatized dog during the first 6 hours after injection of T3. Of the I^{131}, 33% was found in the T3 region, 34% in the X1 (3,3′ T2) region, and 12% in the X2 region. (In the unhydrolyzed urine 23% of the I^{131} was present as T3, and the remainder was found near or below the iodide marker, as in Fig. 3.)

chromatograms. One might speculate that further deiodination occurred with the formation of 3′-monoiodothyronine.

It has been shown previously that tyrosine is conjugated with sulfate, and tyrosine sulfate has been identified in the urine of man (18) and in one of the peptides released from fibrinogen by thrombin (19). Sulfoconjugation of both T3 and Triac has been observed by Roche and coworkers (7–9). Derivatives of these substances now have been shown to be conjugated with sulfate. In the normal dog, sulfoconjugation of T3 and its metabolites occurs to about the same extent as glucuronoconjugation. Storey (20), who has studied the formation of glucuronides and ester sulfates of hydroxyl compounds by liver slices, has suggested that these two processes compete for available phenol acceptors. In dehepatized dogs,

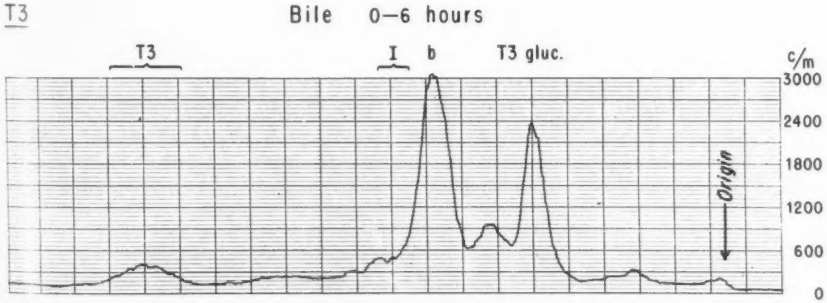

FIG. 7. Radioactivity in a one-dimensional paper chromatogram of bile excreted during the first 6 hours after injection of T3. The total I^{131} in the bile specimen was 27% of the dose. Twenty-two per cent of the biliary I^{131} was in T3 glucuronide. Compound *b* (3,3′ T2 sulfate) accounted for 44% (a small amount of T3 sulfate would be included in this peak). Free T3 accounted for 6%.

most of the T3 or its derivatives are conjugated with sulfate and little, if any, is conjugated with glucosiduronic acid.

When T4 was administered to dogs, only small amounts of the sulfates of T4 or its metabolites were excreted in the bile, and the major products were glucuronides. Closon (9) has found only small amounts of sulfoconjugates in the bile of rats after the administration of T4. In the dehepatized dog, sulfoconjugates and glucuronoconjugates of T4 or its derivatives are excreted in approximately equal amounts.

Previously, we have observed in liverless rats (21) and liverless dogs (22) a great decrease in urinary excretion of I^{131}-labeled inorganic iodide that indicates a decrease in the rate of deiodination of both T3 and T4. Since these thyroidal hormones were labeled only in the 3′ or 5′ positions, deiodination at position 3 or 5 could have occurred without detection. The availability of 3,3′ T2 for use as a standard has now made it possible to show chromatographically that the identical substance is formed as a major metabolite of T3 and that deiodination, therefore, does occur in position 3 or 5. Formation of the metabolite 3,3′ T2 would cause inactivation of T3, according to Gemmill (23) and to Stasilli, Kroc, and Meltzer (24), who have found 3,3′ T2 to be inactive by both calorigenic and antigoitrogenic assays. Particularly after dehepatization in dogs has limited the capacity for removal of iodine from the 3′ position (with formation of radioactive inorganic iodide), 3,3′ T2 accumulates; and this indicates that partial deiodination of the ring in T3 with the alanine side chain is an important pathway in its metabolism. Although extrahepatic tissues thus can remove iodine from one ring of T3 more readily than from the other, Lissitzky and associates (25) have shown that rat liver slices can remove all the iodine atoms from radioactive T4 with the formation of thyronine.

In the presently reported study, 3,3′ T2 was found in only the conjugated form. In earlier studies Roche and co-workers (26) found 3,3′ T2 in rat thyroid, both in the free state and as a constituent of thyroglobulin. The same investigators (27) also found small amounts in the plasma, but Pind (28) found no trace of it in the plasma. 3,3′ T2 has been found in small amounts in the free state in the kidney (29) and muscle (30) of the rat after administration of I^{131}-labeled T3. When I^{131}-labeled 3,3′ T2 was administered to the rat, radioactive inorganic iodide was excreted rapidly in the urine (31).

Acknowledgments

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REFERENCES

1. Taurog, A., F. N. Briggs and I. L. Chaikoff: *J. Biol. Chem.* **194:** 655. 1952.
2. Flock, E. V., J. L. Bollman and J. H. Grindlay: *Am. J. Physiol.* **194:** 33. 1958.

3. Roche, J., O. Michel, R. Michel and J. Tata: *Biochim. et biophys. acta* **13:** 471. 1954.
4. Flock, E. V., J. L. Bollman and J. H. Grindlay: *Am. J. Physiol.* **189:** 420. 1957.
5. Flock, E. V., J. L. Bollman, J. H. Grindlay and B. F. McKenzie: *Endocrinology* **61:** 461. 1957.
6. Gross, J., D. F. Ford, S. Symchowicz and J. H. Horton: The Distribution and Metabolism of Thyroid Hormones. In: Ciba Foundation Colloquia on Endocrinology: Regulation and Mode of Action of Thyroid Hormones. Little, Brown & Company, Boston. Vol. 10, pp. 182–189, 1957.
7. Roche, J., R. Michel, O. Michel and N. Etling: *Comp. rend. Acad. sc.* **245:** 1089. 1957.
8. Roche, J., R. Michel, J. Closon and O. Michel: *Biochim. et biophys. acta* **33:** 461. 1959.
9. Closon, J.: Recherches sur la sulfoconjugaison des hormones thyroidïennes. Thèses de l'Université de Paris. Série A, n° 856; N° d'ordre: 882. Imprimerie Derouaux, Liège, Belgique. 1959.
10. Fauvert, R., J. Roche, R. Michel, P. Thiéblemont and M. Gruson: *Rev. frans., études clinique et biologiques* **3:** 372. 1958.
11. Robinson, D., J. N. Smith, B. Spencer and R. T. Williams: *Biochem. J.* **51:** 202. 1952.
12. Flock, E. V., J. L. Bollman and J. H. Grindlay: *Proc. Staff Meet., Mayo Clin.* **35:** 75. 1960.
13. Grindlay, J. H. and F. C. Mann: *Surgery* **31:** 900. 1952.
14. Roche, J., M. Jutisz, S. Lissitzky and R. Michel: *Biochim. et biophys. acta* **7:** 257. 1951.
15. Roche, J., R. Michel and J. Nunez: *Bull. Soc. chim. biol.* **37:** 809. 1955.
16. Abbott, L. D., Jr.: *Arch. Biochem.* **15:** 205. 1947.
17. Cohen, H. and R. W. Bates: *Endocrinology* **44:** 317. 1949.
18. Tallan, H. H., S. T. Bella, W. H. Stein and S. Moore: *J. Biol. Chem.* **217:** 703. 1955.
19. Bettelheim, F. R.: *J. Am. Chem. Soc.* **76:** 2838. 1954.
20. Storey, I. D. E.: *Biochem. J.* **47:** 212. 1950.
21. Flock, E. V. and J. L. Bollman: *J. Biol. Chem.* **214:** 709. 1955.
22. Flock, E. V., J. L. Bollman, J. H. Grindlay and A. L. Orvis: *Am. J. Physiol.* **187:** 407. 1956.
23. Gemmill, C. L.: *Am. J. Physiol.* **187:** 323. 1956.
24. Stasilli, N. R., R. L. Kroc and R. I. Meltzer: *Endocrinology* **64:** 62. 1959.
25. Lissitzky, S., M.-T. Bénévent, M. Roques and J. Roche: *Compt. rend. Soc. de biol.* **52:** 1490. 1958.
26. Roche, J., R. Michel, W. Wolf and J. Nunez: *Biochim. et biophys. acta* **19:** 308. 1956.
27. Roche, J., R. Michel, J. Nunez and W. Wolf: *Compt. rend Soc. de biol.* **149:** 884. 1955.
28. Pind, K.: *Acta endocrinol.* **26:** 263. 1957.
29. Roche, J., R. Michel, P. Jouan and W. Wolf: *Endocrinology* **59:** 425. 1956.
30. Roche, J., R. Michel and P. Jouan: *Bull. Soc. chim. biol.* **38:** 941. 1956.
31. Roche, J., R. Michel, N. Etling and J. Nunez: *Biochim. et biophys. acta* **22:** 550. 1956.

AUTORADIOGRAPHIC AND HISTOCHEMICAL STUDY OF THE COCK'S COMB IN NORMAL AND HORMONALLY TREATED BIRDS[1]

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ABSTRACT

The cock's comb undergoes histological and histochemical changes with the onset of puberty. Before puberty the corium of the comb is constituted mainly of fibrous connective tissue. After puberty the corium undergoes changes which transforms it into a loose variety of connective tissue with a predominance of ground substance. This ground substance stains metachromatically, gives a positive reaction with Hale's colloidal iron and Alcian Blue and displays an ability for the rapid incorporation of $S^{35}O_4$. These changes increased from the 15th to the 60th day of age.

Testosterone locally applied to the comb of prepuberal birds changed the fibrous type of connective tissue into a mucoid one rich in mucopolysaccharides with and ability to rapidly incorporate $S^{35}O_4$. Castration of the post-puberal bird transformed the mucoid connective tissue to a fibrous type as seen in prepuberal birds. The tests for mucopolysaccharides became less intense or negative and the uptake of $S^{35}O_4$ was diminished. The histochemical reactions as well as the uptake of $S^{35}O_4$ decreased or became negative after treatment with hyaluronidase, or with barium hydroxide extraction, thus showing the presence of a sulfated mucopolysaccharide in the ground substance.

GROWTH of the cock's comb in the Leghorn strain begins at 10–15 days of age and coincides with testicular development and appearance of gonadotrophins in the hypophysis (1, 2, 3). Its growth is regulated by androgenic secretion; parenteral administration or local application of testosterone causes the crest to develop in prepuberal cockerels, while castration brings about its atrophy in adult birds. Atrophy is prevented by administration of testosterone (4, 5). Hypophysectomy of the adult cock produces atrophy of the comb (6), LH appearing to be the gonadotrophin involved (7, 8). Histological and histochemical studies show that the corium of the comb is of the fibrous type from birth to puberty. After puberty it develops an intermediate mucoid zone rich in ground substance which contains hyaluronic acid (5), glucosamine and a high content of water (9).

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This work is designed to show the presence in the mucoid zone of another mucopolysaccharide, chondroitinsulfuric acid as well as hyaluronic acid.

MATERIAL AND METHODS

Untreated animals. White Leghorn cocks of 5, 15, 30 and 60 days of age were used, 16 birds being placed in each age group. After a biopsy of one peak of the comb each bird received an intraperitoneal injection of sodium sulfate labeled with S^{35}. The dose employed was two microcuries per gram of body weight. Subsequent biopsies of combs were taken at 15 minutes, 30 minutes, 1, 2, 5, 12, 24, 48 and 72 hours and then every three days up to a period of 21 days.

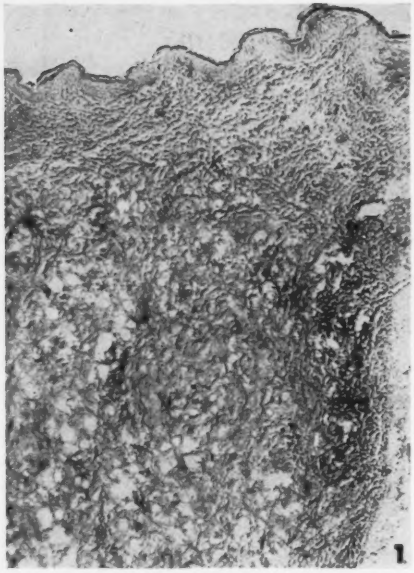
Treated animals. These were divided into six groups. Groups I and II each consisted of 14 prepuberal cockerels. Testosterone in sesame oil (100 mg. in 0.01 ml.) was applied locally to the combs of Group II for 8 days. Group I served as controls and received only sesame oil. Groups III, IV, V and VI each consisted of 10 adult birds of 90 days of age. Groups IV, V and VI were castrated. After one month intramuscular injections of 2 mg. daily (for 10 days) of testosterone propionate were started in Group V, and after two months in Group VI. Groups III and IV were used as intact and castrated controls. All birds were injected intraperitoneally with 2 μc. of $S^{35}O_4$ per gm. of body weight in the form of sodium sulfate. Removal of the peaks of the comb was begun at one hour after the injection and continued at 2-hour intervals for 24 hours.

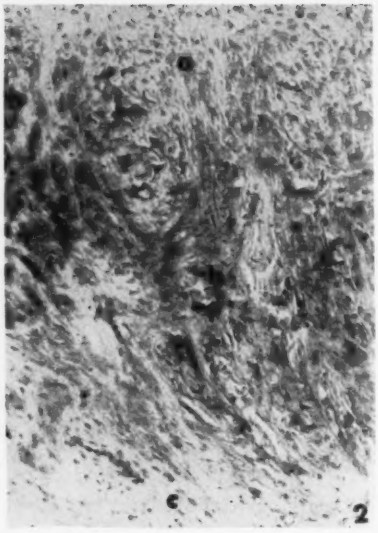
Tissues were fixed in Bouin's and Carnoy's fluids and in 10% formalin and sections cut at 5, 10 and 20 μ. Some sections were incubated in testicular hyaluronidase and some in a saturated solution of barium hydroxide. Pelc's stripping film method was used for autoradiography (11). The following techniques were used for histogical and histochemical purposes: hematoxylin and eosin, Azan and van Gieson for routine observations, orcein for elastic fibers, silver impregnation for reticular fibers. Mucopolysaccharides were demonstrated by the metachromatic reaction (toluidine blue 0.25%, pH 4), Hale's colloidal iron, Alcian Blue and Periodic Acid Leucofuchsin (PAS). The specificity of the latter three techniques was controlled through incubation of the sections in testicular hyaluronidase as already indicated.

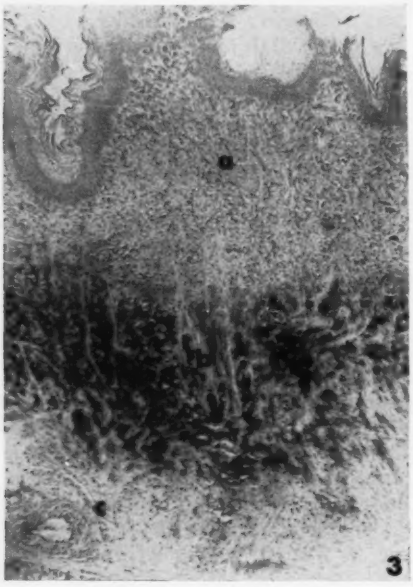
The determinations of the concentrations of $S^{35}O_4$ in the histological sections were made by comparison with reference standards. For the evaluation of the blackening produced in the stripping film by the Beta particles emitted by the $S^{35}O_4$ present in the specimen, a microdensitometer of high sensitivity was employed. The densitometer consisted of an optical system (phase contrast microscope and a photographic enlarger with lateral prism) and Du Mont 6467 and RCA 1 P 21 photomultipliers. An adjustable diaphragm was used for delimitation of the sensitivity area of the photocathode and the area to be measured. The linear scanning of the image was achieved with two motors displacing the photomultipliers and accessories in synchronization with the recording system (General Electric Recorder CE). The appropriate sensitivity of the scale was obtained with an attenuator and multiplier circuit. The photometric lamp was fed with a stablilizing source of 6 volts ($\pm$0.01 volts). This apparatus permitted work in a range of optical density between 0.01 and 1.0. For various types of tissues and for thickness varying from 1 to 10 μ the optical index found was of $\pm$0.03. The amount of visible light absorbed by the autoradiograph was related to the total number of silver grains developed per area unit.

RESULTS

Untreated birds. In untreated birds 5 days of age the corium was entirely of the fibrous type with very little ground substance (Fig. 1). In birds 15







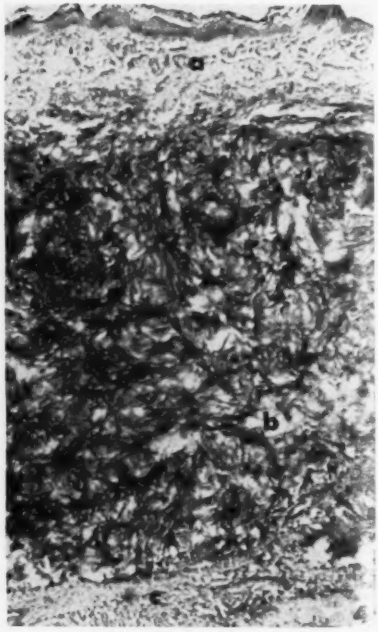


FIG. 1. Comb of a 5-day old male chick. Hale's colloidal iron technique, showing a negative result all through the connective tissue. ×150.

FIG. 2. Comb of a 15-day old cock. Hale's colloidal iron technique, showing scarce positive material in some areas of the intermediate layer. ×150.

FIG. 3. Comb of a 30-day old cock. Hale's colloidal iron technique, showing moderate positive reaction in the intermediate layer. ×150.

FIG. 4. Comb of an adult cock (approx. 60 days). Hale's colloidal iron technique, showing intensely positive material in the intermediate layer. ×150.

a = superficial fibrous layer
b = intermediate mucoid layer
c = fibrovascular central zone

o 60 days of age the corium consisted of a superficial fibrous layer and n intermediate or mucoid layer and a fibrovascular central core (Figs. 2, 3, 4).

In birds 15 days of age and older the ground substance in the intermediate zone showed a positive reaction with Alcian Blue and Hale's colloidal iron (Figs. 2, 3, 4) and the metachromasia was positive, demonstrating a mucopolysaccharide content increasing progressively with age. The PAS technique and silver impregnation demonstrated reticular and collagen fibers. In the superficial and central layers the reaction for mucopolysaccharides was positive only in the walls of the vessels. The PAS reaction and silver impregnation showed reticular and collagen fibers and the basement membrane, Treatment with hyaluronidase removed completely the staining of mucopolysaccharides in both fibrous and mucoid layers, but did not affect the PAS and silver impregnation tests for collagen and rectiular fibers and for the basement membrane. The uptake of $S^{35}O_4$ was negative in the 5-day old birds (Fig. 5) but became progressively positive in birds from 15 to 60 days of age (Figs. 6, 7, 8). In the first hours after the injection, a transitory uptake of $S^{35}O_4$ was visible in the cytoplasm of the fibroblasts and in the area immediately surrounding them (Fig. 17). After 24 hours the distribution became extracellular (Fig. 18) and appeared predominantly in the intermediate layer corresponding with the histochemical picture for mucopolysaccharides. In the superficial and central layers a very slight intercellular uptake of $S^{35}O_4$ was visible. The walls of the arteries and veins showed a moderate uptake.

Figure. 19 shows in semi-logarithmic scale the concentration of $S^{35}O_4$ in the sections of the combs and the variations with time in the four groups of different ages. The incubation of these sections in hyaluronidase and barium hydroxide showed that the extraction of $S^{35}O_4$ was complete with barium hydroxide and only partial with hyaluronidase.

Treated birds. In prepuberal birds local application of testosterone promoted within a few days a general growth of the comb, with the development of an intermediate zone with histochemically demonstrable mucopolysaccharide (cf. Figs. 9 and 10) and an intense uptake of $S^{35}O_4$ (cf. Figs. 13 and 14). Castration in adult cocks produced atrophy of the comb, decreased the content of mucopolysaccharides (Fig. 11) and reduced the extent of uptake of $S^{35}O_4$ in the mucoid layer (Fig. 15). Administration of testosterone to castrated birds promoted within a few days the growth of the comb and produced similar histological, histochemical and autoradiographic aspects of the mucoid zone to those of the prepuberal birds treated with testosterone (Figs. 12, 16). Figure 20 shows the densitometric curves for the four groups of treated birds and their corresponding controls.

DISCUSSION

This work indicates a close relationship between the appearance and increase of the mucopolysaccharide content and the progressive uptake of

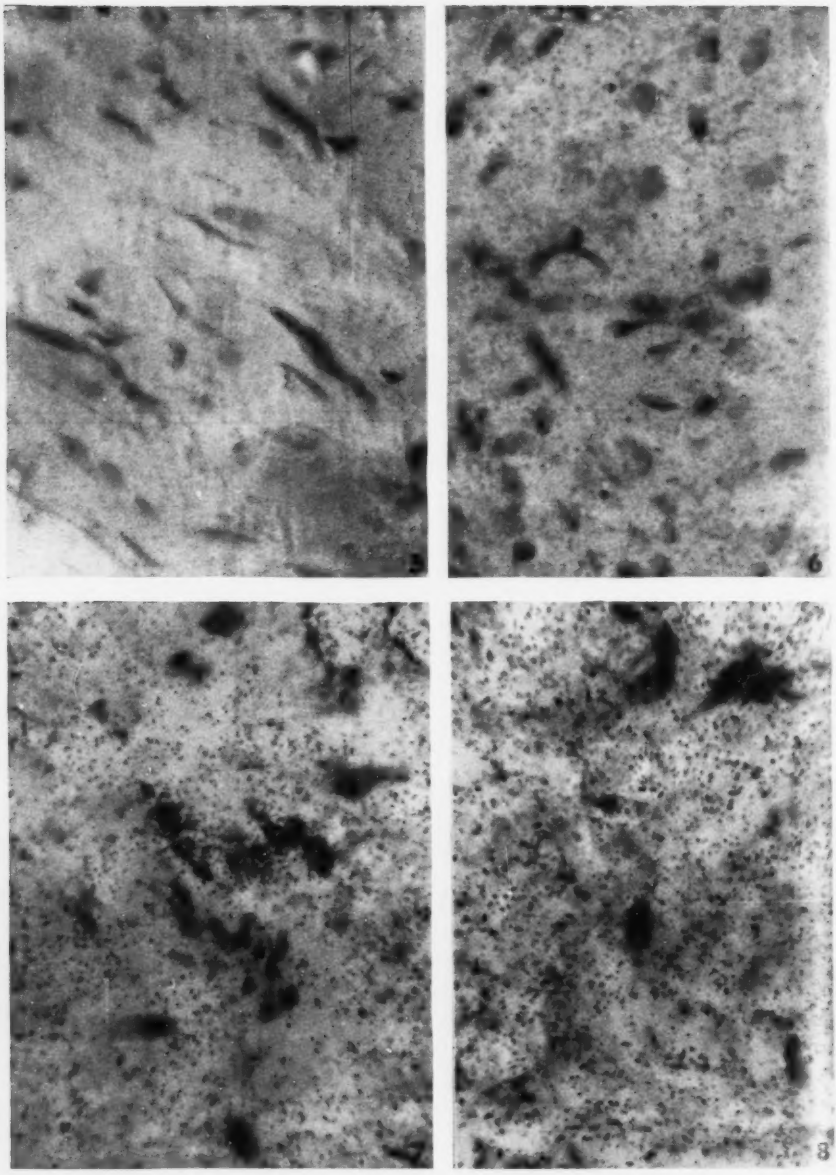
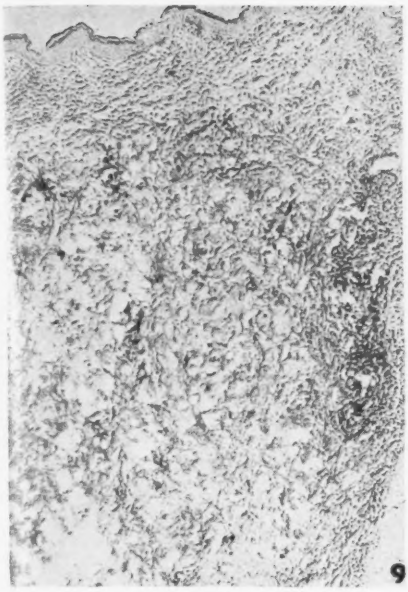


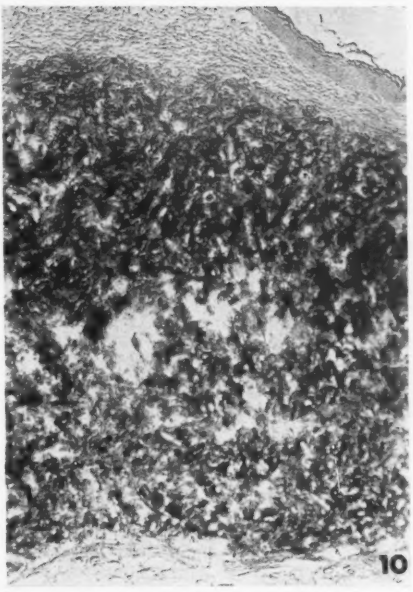
FIG. 5. Comb of a 5-day old male chick. The autoradiograph is negative in the connective tissue, 24 hours after the administration of $S^{35}O_4$. ×800.

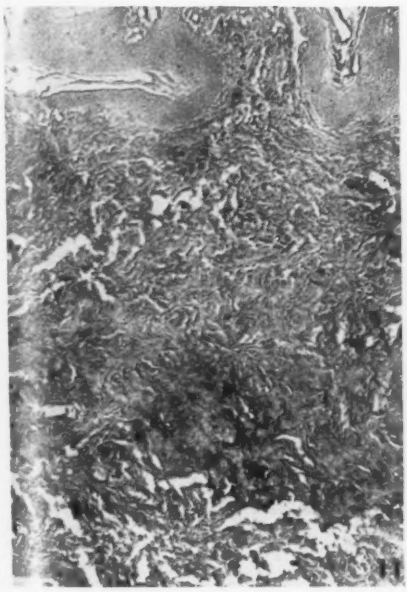
FIG. 6. Comb of a 15-day old cock. Autoradiograph of specimen removed 24 hours after administration of $S^{35}O_4$. There is a slight uptake of $S^{35}O_4$ in the intermediate layer mainly in the intercellular spaces. ×800.

FIG. 7. Comb of a 30-day old cock. Autoradiograph of specimen removed 24 hours after administration of $S^{35}O_4$. There is a moderate uptake of $S^{35}O_4$ in the intermediate layer mainly in the intercellular spaces. ×800.

FIG. 8. Comb of an adult cock (approx. 60 day). Autoradiograph of specimen removed 24 hours after the administration of $S^{35}O_4$. There is an intense uptake of $S^{35}O_4$ in the intermediate layer mainly in the intercellular spaces. ×800.







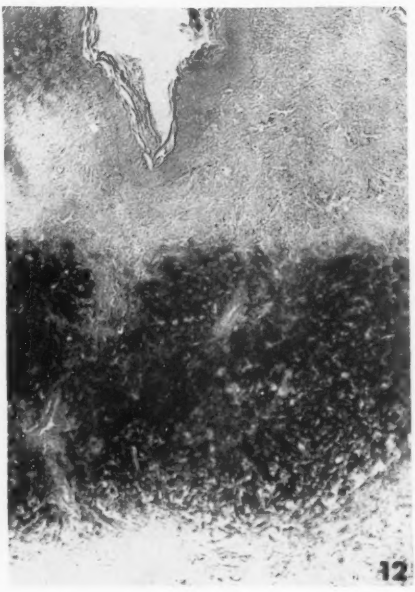
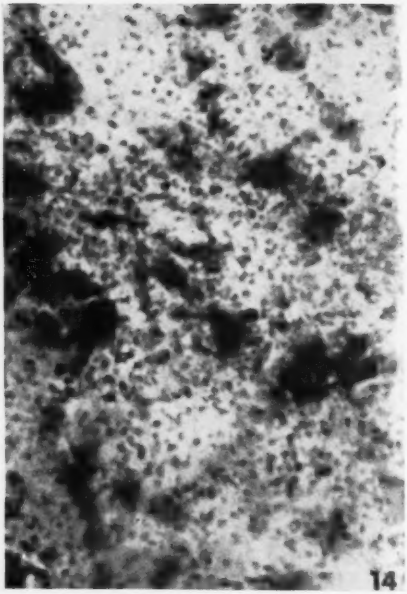


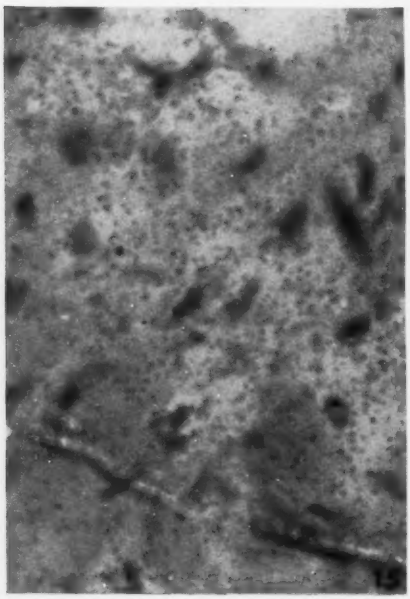
FIG. 9. Comb of a 5-day old male chick. Hale's colloidal iron technique. The reaction is negative throughout the connective tissue. ×150.

FIG. 10. Comb of a 5-day old male chick, treated locally with testosterone. Hale's colloidal iron technique. The reaction is strongly positive in the intermediate layer (compare with Fig. 1). ×150.

FIG. 11. Comb of a castrate adult cock. Hale's colloidal iron technique. The reaction is weakly positive in the intermediate layer. ×150.

FIG. 12. Comb of the adult castrate cock, treated with injections of testosterone. Hale's colloidal iron technique. The reaction is intensely positive in the intermediate layer. ×150.





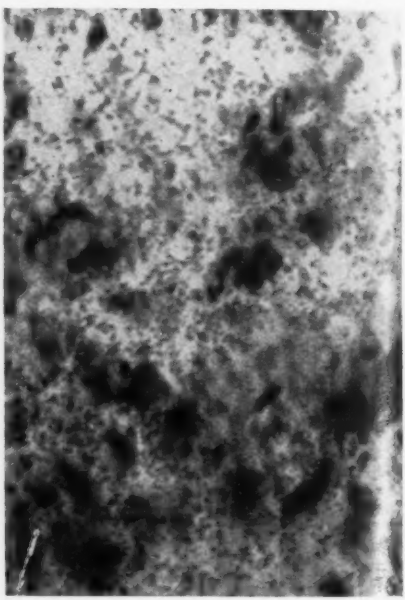


FIG. 13. Comb of a 5-day old male chick, 24 hours after the administration of $S^{35}O_4$. The autoradiograph is negative in the connective tissues. ×800.

FIG. 14. Comb of a 5-day old male chick, treated locally with testosterone. 24 hours after the administration of $S^{35}O_4$. The autoradiograph shows an intense uptake of $S^{35}O_4$ in the connective tissue of the intermediate layer. ×800.

FIG. 15. Comb of the adult castrate cock, 24 hours after the injection of $S^{35}O_4$. The autoradiograph shows a slight uptake in the connective tissue. ×800.

FIG. 16. Comb of an adult castrate cock, treated with testosterone injections. The autoradiograph of the specimen removed 24 hours after the injection of $S^{35}O_4$, shows an intense uptake of the isotope. ×800.

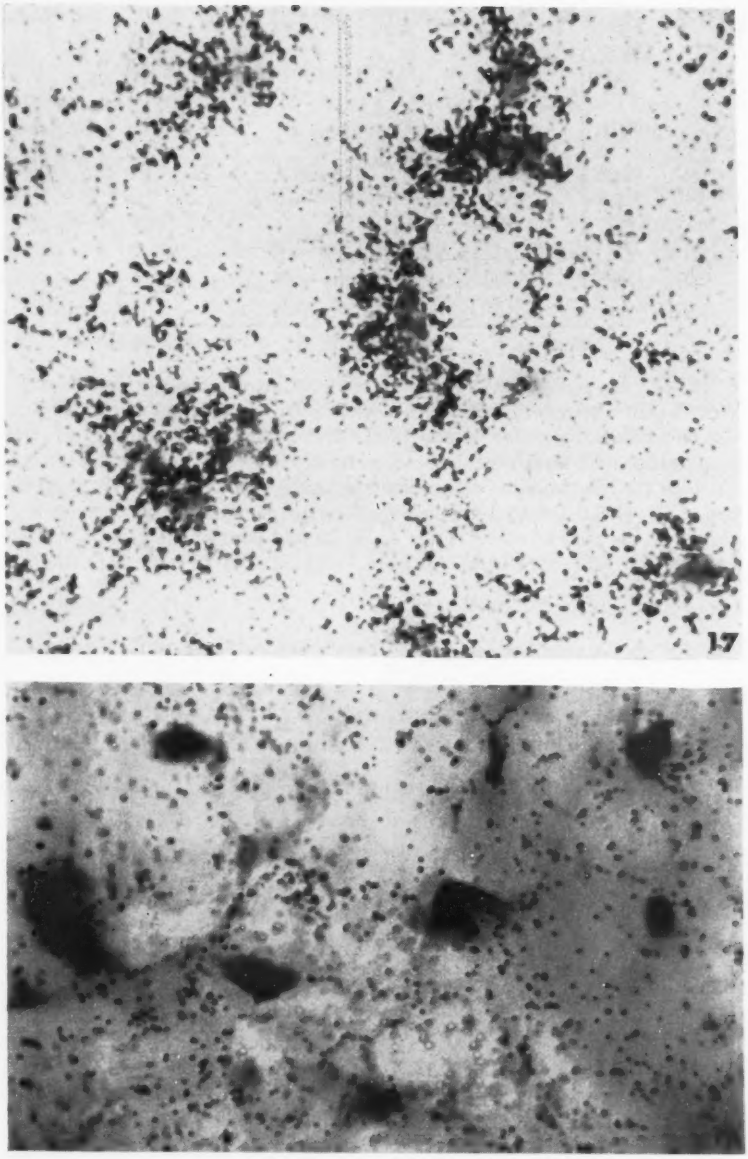


FIG. 17. Intermediate layer of the comb of a 6-day old male chick, treated for 8 days with testosterone locally applied, one hour after intraperitoneal injection of the isotope. The uptake has occurred mainly in the cytoplasm of the fibroblasts. A moderate uptake of $S^{35}O_4$ is also visible in the ground substance around the cells. ×950.

FIG. 18. Section of the comb taken from the same bird in Figure 17 except that the tissue was removed 24 hours after the injection of $S^{35}O_4$. The distribution of the isotope is now mainly in the ground substance. ×950.

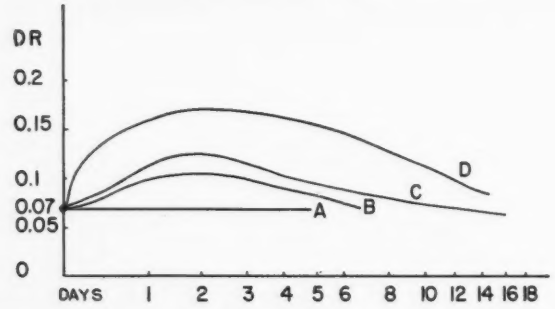


FIG. 19. Graphic representation of the variations in radioautographic density of the cock's comb after intraperitoneal injection of $S^{35}O_4$, at different ages and intervals. D-R signifies radioautographic density of the histological sections and is a function of the concentration of $S^{35}O_4$ in the intermediate layer of the combs. *0.07:* This is a constant factor due to the absorption of the radioautographic film and the particular structure analyzed. A, B, C and D represent the densitometric curves corresponding to the autoradiographs of the cock's combs of the 5, 15, 30 and 60 day age groups respectively. For each age the values are expressed from 15 minutes to 18 days after the injection of $S^{35}O_4$.

$S^{35}O_4$ in the cock's comb under androgenic stimulation. The growth of the intermediate mucoid zone at puberty is accompanied by the appearance of mucopolysaccharides and a corresponding increased uptake of $S^{35}O_4$ in the mucoid layer. The tests made with barium hydroxide and hyaluronidase

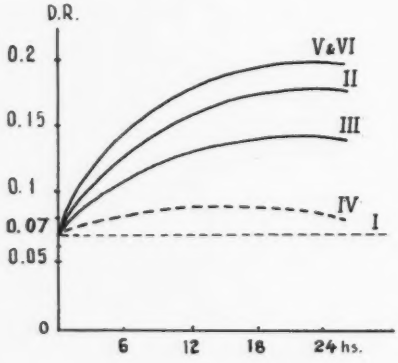


FIG. 20. Graphic representation of the variations in the autoradiographic density of the cock's comb after intraperitoneal injection of $S^{35}O_4$, under the experimental conditions detailed in the text. D-R signifies autoradiographic density of the histological sections and is the function of the concentration of $S^{35}O_4$ in the mucoid layer of the comb. 0.07 represents a constant factor due to the absorption of the autoradiographic film and the particular structure analyzed.

I = Group I: Prepuberal untreated birds (control for Group II)
II = Group II: Prepuberal birds, treated for 8 days with testosterone applied locally.
III = Adult untreated birds.
IV = Adult castrate birds (control for Groups V and VI).
V & VI = Adult castrate birds, treated for 10 days with testosterone, injected intramuscularly. The injections started one month after castration for Group V and two months after castration for Group VI.

show that the substance responsible for the uptake of $S^{35}O_4$ is a sulfated mucopolysaccharide which has already been demonstrated in other types of connective tissue (12). The presence of chondroitin sulphuric acid has been shown biochemically in the comb of adult cocks (13). Another mucopolysaccharide, hyaluronic acid, which is also androgen dependent, has been demonstrated in the cock's comb (5). The hormonal dependence of these mucopolysaccharides is further substantiated by the finding that the level of hexosamine is low in prepuberal and castrated adult cocks, and attains normal adult values after the administration of testosterone (9, 10).

The possible role of the fibroblasts in the production of this polysaccharide is suggested by their increased basophilia and cytoplasmic alkaline phosphatase (9), their hypertrophy and the accumulation of $S^{35}O_4$ within and around the cell bodies during the first few hours after the injection of the isotope. These findings point to the ability of the fibroblasts of the cock's comb to respond to the stimulus of androgenic hormones, and therefore to their active participation in the process of mucoid transformation of fibrous connective tissue.

The uptake of the radioisotope by the fibrous connective tissue of the subepithelial corium and the central core of the comb is far less intense than that of the mucoid layer. The uptake of isotope by these zones also increases from puberty to adulthood and is stimulated by the action of testosterone. As histochemical techniques for mucopolysaccharides give negative results in these zones, the greater sensitivity of the radioautographic method with $S^{35}O_4$ confirms the existence of sulfated polysaccharides in this fibrous type of connective tissue as has been shown in other species (14, 15).

The metabolism of mucopolysaccharides in the mucoid zone, as measured with $S^{35}O_4$ is more rapid in the 15-day old birds than in those of 30 and 60 days of age. In the former group the autoradiographs became negative 8 days after the injection of the radioisotope, while in the latter it disappeared from the tissue after 18 days.

These facts indicate that the metabolism of the sulfated mucopolysaccharides in the mucoid zone is relatively slow, as it is in some other types of connective tissue and cartilage (16, 17).

REFERENCES

1. Champy, C. and N. Kritch: *C. Rend. Soc. Biol.* **92:** 683. 1925.
2. Champy, C. and M. Demay: *C. Rend. Soc. Biol.* **119:** 23. 1935.
3. Hardesty, W.: *Am. J. Anat.* **47:** 277. 1931.
4. Szirmai, J. A.: *Anat. Rec.*, **105:** 337. 1949.
5. Boas, N. F.: *J. Biol. Chem.* **181:** 573. 1949.
6. Nalbandov, A. F., H. R. Meyer and W. Moshan: *Endocrinology* **39:** 91. 1946.
7. Nalbandov, A. F. and L. Card: *Endocrinology* **38:** 71. 1946.
8. Breneman, W. R.: *Anat. Rec.* **117:** 533. 1953.

9. Szirmai, J. A.: *J. Histoch. and Cytoch.* **4:** 97. 1956.
10. Ludwig, A. and N. F. Boas: *Endocrinology* **46:** 291. 1950.
11. Pelc, S. R.: *Nature* **160:** 749. 1947.
12. Mancini, R. E., C. Nunez and E. S. Lustig: *Rev. Soc. Arg. Biol.* **31:** 322. 1955.
13. Dorfman, A. and S. Schiller: *Proc. Soc. Exp. Biol. Med.* **92:** 100. 1956.
14. Davies, D. V. and L. Young. *J. of Anat.* **88:** 179. 1954.
15. Mancini, R. E. and E. S. Lustig: *Rev. Soc. Arg. Biol.* **30:** 67. 1954.
16. Pelc, S. R. and A. Glucksmann: *Exp. Cell Res.* **8:** 336. 1955.
17. Belanger, L. F.: *Anat. Rec.* **88:** 174. 1954.

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